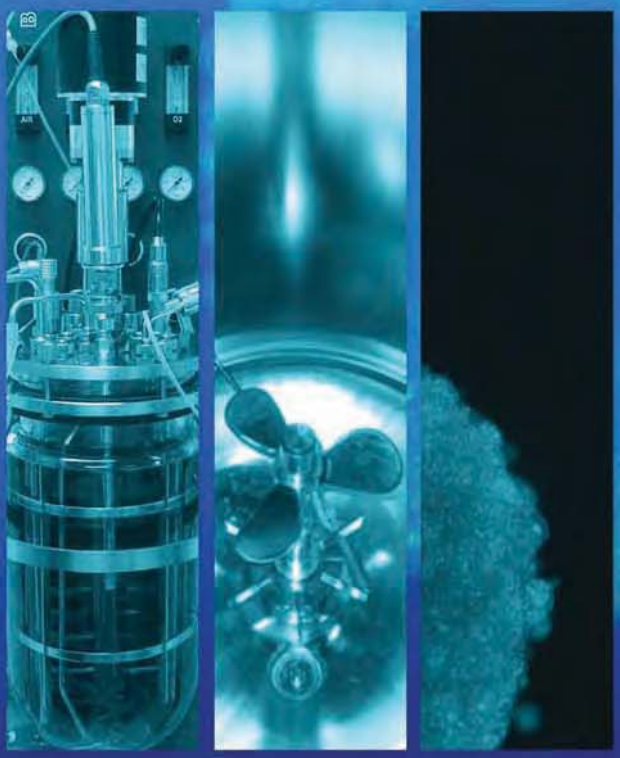
Animal Cell Technology:
From Biopharmaceuticals to Gene Therapy

Edited by
Leda R. Castilho, Ângela Maria Moraes, Elisabeth F. P. Augusto and Michael Butler



Animal Cell Technology: From Biopharmaceuticals to Gene Therapy

Animal Cell Technology: From Biopharmaceuticals to Gene Therapy

Edited by

Leda R. Castilho

Cell Culture Engineering Laboratory, Chemical Engineering Program – COPPE, Federal University of Rio de Janeiro, Brazil

Ângela Maria Moraes

School of Chemical Engineering, Department of Biotechnological Processes, State University of Campinas, Brazil

Elisabeth F.P. Augusto

Institute of Technological Research of the State of São Paulo, Brazil

and

Michael Butler

Department of Microbiology, University of Manitoba, Canada



Taylor & Francis
Taylor & Francis Group

Published by:

Taylor & Francis Group

In US: 270 Madison Avenue
New York, N Y 10016

In UK: 2 Park Square, Milton Park
Abingdon, OX14 4RN

This edition published in the Taylor & Francis e Library, 2008.

"To purchase your own copy of this or any of Taylor & Francis or Routledge's collection of thousands of eBooks please go to www.eBookstore.tandf.co.uk."

© 2008 by Taylor & Francis Group

ISBN: 978 0 415 42304 5

This book contains information obtained from authentic and highly regarded sources. Reprinted material is quoted with permission, and sources are indicated. A wide variety of references are listed. Reasonable efforts have been made to publish reliable data and information, but the author and the publisher cannot assume responsibility for the validity of all materials or for the consequences of their use.

All rights reserved. No part of this book may be reprinted, reproduced, transmitted, or utilized in any form by any electronic, mechanical, or other means, now known or hereafter invented, including photocopying, microfilming, and recording, or in any information storage or retrieval system, without written permission from the publishers.

A catalog record for this book is available from the British Library.

Library of Congress Cataloging in Publication Data

Library of Congress Cataloging in Publication Data
Animal cell technology : from biopharmaceuticals to gene therapy / edited by Leda R. Castilho ... [et al.].
p. ; cm.
Includes bibliographical references and index.
ISBN 978 0 415 42304 5 (alk. paper)
1. Animal cell biotechnology. I. Castilho, Leda dos Reis.
[DNLM: 1. Cells, Cultured. 2. Animals. 3. Biotechnology. 4. Cell Culture Techniques veterinary. QU 300 A598 2008]
TP248.27.A53A5453 2008
660.6 dc22

2007042273

ISBN 0-203-89516-9 Master e-book ISBN

Senior Editor: Elizabeth Owen
Editorial Assistant: Kirsty Lyons
Senior Production Editor: Simon Hill

10 9 8 7 6 5 4 3 2 1

T&F informa

Taylor & Francis Group, an informa business

Visit our web site at http://www.garlandscience.com

Contents

Contributors xiv

Abbreviations xvi

Foreword xxxv

1 Introduction to animal cell technology **1**
Paula Marques Alves, Manuel José Teixeira Carrondo, and Pedro Estilita Cruz
1.1 Landmarks in the culture of animal cells 1
1.2 Types of animal cell cultures 3
1.3 Use of animal cells in commercial production 5
1.3.1 Animal cell proteins in human diagnosis and therapy 5
1.3.2 Cell therapy 7
1.3.3 Tissue engineering 8
1.3.4 Gene therapy and DNA vaccines 9
1.3.5 Applications of animal cells in the development of new products 9
1.4 Conclusions 10
References 11

2 Animal cells: basic concepts **13**
Patrícia Léo, Adriana Lages Lima Galesi, Cláudio Alberto Torres Suazo, and Ângela Maria Moraes
2.1 Introduction 13
2.2 Typical structure of an animal cell 13
2.2.1 Plasma membrane 14
2.2.2 Cytoplasm 15
2.2.3 Endoplasmic reticulum 15
2.2.4 Ribosome 16
2.2.5 Golgi complex 16
2.2.6 Mitochondria 16
2.2.7 Lysosome 16
2.2.8 Peroxisome 17
2.2.9 Nucleus 17
2.3 Cell culture 17
2.3.1 Establishing a cell line 17
2.3.2 Cell line maintenance 20
2.4 Cell growth phases 21
2.5 Influence of environmental conditions on animal cell culture 24
2.5.1 pH 24
2.5.2 Osmolality 25
2.5.3 Temperature 26
2.5.4 Oxygen supply 26

2.5.5 Composition and nature of the substratum for cell adhesion 27
2.6 Cryopreservation and storage of cell lines 28
2.7 Culture quality control and laboratory safety 29
2.8 Characteristics of the main cell lines employed industrially 30
2.9 Culture of insect cells 31
2.10 Use of animal cell culture in cytotoxicity assays 32
2.10.1 Culture methods 33
2.10.2 Exposure time and active agent concentrations 34
2.10.3 Recovery time 35
2.10.4 Cytotoxicity evaluation methods 35
References 36

3 Cloning and expression of heterologous proteins in animal cells 39
Mariela Bollati-Fogolín and Marcelo A. Comini
3.1 Introduction 39
3.2 The flow of genetic information and molecular cloning 39
3.3 Elements required for gene expression in eukaryotic cells 40
3.3.1 Transcriptional control elements 40
3.3.2 Translational control elements 42
3.4 Systems for heterologous expression in animal cells 44
3.4.1 Viral vectors 44
3.4.2 Baculoviruses 48
3.4.3 Plasmid vectors 50
3.5 Cell lines and biotechnological processes 54
3.6 Expression in animal cells 54
3.6.1 Transient expression 55
3.6.2 Stable expression 56
3.7 Introduction of DNA into mammalian cells 58
3.7.1 Calcium phosphate co-precipitation method 58
3.7.2 Cationic polymers 59
3.7.3 Lipid-mediated gene transfer (lipofection) 60
3.7.4 Electroporation 60
3.8 Selection markers 61
3.8.1 Morphological changes 61
3.8.2 Biochemical markers and gene amplification 61
3.8.3 Reporter markers 64
3.9 Screening, quantitation, and bioassay methods 66
3.10 Optimizing the initial stage of an animal cell-based bioprocess 66
References 67

4 Cell metabolism and its control in culture 75
Paola Amable and Michael Butler
4.1 Introduction 75
4.2 Energy sources 76
4.2.1 Glucose 76
4.2.2 Glutamine 84
4.2.3 Amino acids 87
4.2.4 Lipids 91

4.3 Metabolic byproducts 95
4.3.1 Lactate 95
4.3.2 Ammonia 96
4.4 Factors affecting cell metabolism 101
4.4.1 Oxygen requirements 102
4.4.2 Carbon dioxide 103
4.4.3 Temperature 103
4.4.4 pH 104
4.5 Conclusions 104
References 104

5 Culture media for animal cells 111
Ângela Maria Moraes, Ronaldo Zucatelli Mendonça, and Claudio Alberto Torres Suazo
5.1 Introduction 111
5.2 Main components of animal cell culture media 114
5.2.1. Water 114
5.2.2 Glucose 115
5.2.3 Amino acids 116
5.2.4 Vitamins 117
5.2.5 Salts 117
5.2.6 Serum 117
5.2.7 Other components necessary for cell culture 118
5.3 Advantages and limitations of the use of media supplemented with animal serum 121
5.4 Strategies to formulate serum-free culture media 122
References 125

6 Post-translational modification of recombinant proteins 129
Michael Butler
6.1 Introduction 129
6.2 Glycan structures attached to proteins 130
6.2.1 N-glycans 130
6.2.2 O-linked glycans 133
6.2.3 Patterns of glycosylation in nonmammalian cells 134
6.2.4 Glycosylation in animal cells: the effect of the host cell line 137
6.2.5 Culture parameters that may affect glycosylation 137
6.3 Other forms of post-translational modification 138
6.3.1 Deamidation 138
6.3.2 Deamination 139
6.3.3 Glycation 139
6.3.4 Gamma-carboxylation 140
6.3.5 C-terminal modifications 142
6.3.6 Hydroxylation 142
6.4 Conclusions 142
Acknowledgments 143
References 143

7 Mechanisms of cell proliferation and cell death in animal cell culture *in vitro* 147
Maíra Peixoto Pellegrini, Rodrigo Coelho Ventura Pinto, and Leda dos Reis Castilho
7.1 Introduction 147
7.2 Cell proliferation mechanisms 147
7.3 Cell death mechanisms: apoptosis and necrosis 151
7.4 Influence of environmental conditions on the induction of cell death 152
7.4.1 Depletion of nutrients and growth factors 152
7.4.2 Oxygen limitation 154
7.4.3 Susceptibility to shear stress 154
7.4.4 Osmolality 155
7.5 Methods of detection of cell death by apoptosis 155
7.5.1 DNA fragmentation 156
7.5.2 Morphological changes 157
7.5.3 Membrane asymmetry 158
7.5.4 Apoptotic proteins 158
7.5.5 Cytochrome C release 159
7.6 Apoptosis suppression by molecular techniques 159
7.6.1 Molecular basis of apoptotic cell death 159
7.6.2 Molecular strategies for apoptosis control 171
7.7 Conclusions and perspectives 173
References 173

8 Mathematical models for growth and product synthesis in animal cell culture 181
Elisabeth F.P. Augusto, Manuel F. Barral, and Rosane A.M. Piccoli
8.1 Introduction 181
8.2 Kinetic analysis of bioprocesses 185
8.2.1 Characteristic kinetic variables 186
8.2.2 Data treatment 190
8.2.3 Phenomena identification 191
8.3 Unstructured and nonsegregated models 192
8.3.1 Classical formulas for cell growth, substrate consumption, and product synthesis 192
8.3.2 Kinetic models for animal cells 199
8.3.3 Parameter fitting in models 209
8.3.4 Model validation 213
8.4 Structured and nonsegregated models 214
8.5 Unstructured and segregated models 215
References 218

9 Bioreactors for animal cells 221
Ernesto Chico Véliz, Gryssell Rodríguez, and Alvio Figueredo Cardero
9.1 Introduction 221
9.2 Inoculum propagation and small-scale culture systems 221
9.3 Types of bioreactors 224
9.3.1 Homogeneous bioreactors 225
9.3.2 Heterogeneous bioreactors 228
9.4 Modes of operation of bioreactors 234
9.4.1 Batch cultivation 235

9.4.2 Fed-batch cultivation 237
9.4.3 Continuous cultivation 240
9.4.4 Continuous cultivation with cell retention (perfusion) 242
9.5 Aeration and agitation 246
9.6 Scale-up 250
9.7 Economic aspects relevant to bioreactor selection: the productivity factor 252
References 255

10 Monitoring and control of cell cultures 259
Aldo Tonso
10.1 Introduction 259
10.2 Monitoring and control: basic concepts 259
10.3 Particular characteristics of cell cultures 261
10.4 Main bioprocess variables 261
10.4.1 Temperature 261
10.4.2 pH 262
10.4.3 Dissolved oxygen 263
10.4.4 Cell concentration 265
10.4.5 Other variables of interest 267
10.5 Strategies of control 268
10.5.1 Traditional control 268
10.5.2 Advanced control 270
References 270

11 Animal cell separation 273
Leda dos Reis Castilho and Ricardo de Andrade Medronho
11.1 Introduction 273
11.2 Separation efficiency 274
11.3 Gravity settling 280
11.4 Centrifugation 281
11.5 Hydrocyclones 283
11.6 Filtration 285
11.6.1 Tangential flow filtration with membranes 285
11.6.2 Dynamic filters 287
11.6.3 Spin-filters 288
11.7 Ultrasonic separation 289
References 291

12 Product purification processes 295
Ângela Maria Moraes, Leda dos Reis Castilho, and Sônia Maria Alves Bueno
12.1 Introduction 295
12.2 Basic considerations 295
12.2.1 Final application of product 296
12.2.2 Selection of the protein source 297
12.2.3 Protein properties and manipulation 298
12.3 Cell disruption 298
12.4 Protein purification methods 300
12.4.1 Separation processes based on solubility 301
12.4.2 Separation processes based on differences in molar mass 304

12.4.3 Separation processes based on differences in electrical charge 309
12.4.4 Separation processes based on differences in hydrophobicity 313
12.4.5 Separation processes based on specificity of ligands 314
12.4.6 Other developments 319
12.5 Conclusions 323
References 324

13 Quality control of biotechnological products 329
Marina Etcheverrigaray and Ricardo Kratje
13.1 Introduction 329
13.2 Production of recombinant proteins 331
13.2.1 Control of starting materials 331
13.2.2 Quality control of cell banks 333
13.3 Control of the production process 334
13.3.1 Cultures 334
13.3.2 Purification 335
13.4 Product control 335
13.4.1 Characterization and specification 335
13.4.2 Protein content 336
13.4.3 Amino acids analysis (identification and/or protein content) 336
13.4.4 Protein sequencing (identification) 337
13.4.5 Peptide mapping 337
13.4.6 Electrophoresis 337
13.4.7 Carbohydrate determination 340
13.4.8 Potential impurities and contaminants of biotechnological products 340
13.5 Bioassays 341
13.5.1 Bioassay types 342
13.5.2 *In vitro* bioassays 343
13.5.3 Experimental design 344
13.5.4 Statistical analysis 345
References 345

14 Regulatory aspects 349
Maria Teresa Alves Rodrigues and Ana Maria Moro
14.1 Introduction 349
14.2 Good Manufacturing Practices and quality assurance 350
14.3 Regulatory agencies 351
14.4 Harmonization 352
14.5 Premises 353
14.5.1 Clean rooms 353
14.5.2 Biosafety 354
14.6 Cell banks 355
14.6.1 Cell bank qualification 355
14.7 Validation 358
14.7.1 General aspects 358
14.7.2 Biological products 360
14.8 Stability 362
14.9 Clinical trials 362
14.9.1 Preclinical studies 363

14.9.2 Clinical studies 364
14.10 Biogenerics or biosimilars 365
References 367

15 Intellectual property 373
Ana Cristina Almeida Müller and Leila Costa Duarte Longa
15.1 Introduction 373
15.2 The biotechnology sector 373
15.3 Ethical and moral aspects of research involving genetic engineering 374
15.4 Basic concepts of patentability 376
15.4.1 Discovery versus invention 376
15.4.2 Requirements for the patentability of inventions 377
15.5 Patentable materials 382
15.6 Industrial property and technology transfer offices 384
15.7 Patent and technology transfer specialists 386
15.8 Conclusions 388
References 388

16 Recombinant therapeutic proteins 389
Maria Candida Maia Mellado and Leda dos Reis Castilho
16.1 Introduction 389
16.2 Main therapeutic proteins 389
16.2.1 Cytokines 390
16.2.2 Hematopoietic growth factors 392
16.2.3 Growth factors 392
16.2.4 Hormones 393
16.2.5 Therapeutic enzymes 393
16.2.6 Blood coagulation factors 398
16.2.7 Antibodies 399
16.3 Economic aspects 400
16.4 Challenges and future perspectives 402
16.4.1 Formulation and delivery of biopharmaceuticals 402
16.4.2 Characterization of biopharmaceuticals 404
16.4.3 Alternative expression systems 404
16.4.4 Second-generation biopharmaceuticals 405
References 406

17 Monoclonal antibodies 409
Wirla M.S.C. Tamashiro and Elisabeth F.P. Augusto
17.1 Introduction 409
17.2 Antibodies 411
17.3 Production of monoclonal antibodies 415
17.3.1 Step 1: Immunization 415
17.3.2 Step 2: Fusion and selection of secreting hybridomas 416
17.3.3 Step 3: Hybridoma cloning 417
17.3.4 Step 4: Definition of the isotype of monoclonal antibodies obtained 417
17.3.5 Step 5: Follow-up/later developments 417
17.4 Production of recombinant antibodies 418

17.4.1 Humanized antibodies 420
17.4.2 Human antibodies 421
17.5 Production systems 425
17.5.1 Cell lines 426
17.5.2 Basic conditions for *in vitro* cultivation 427
17.5.3 Cell metabolism 428
17.5.4 Bioreactors and operation mode 429
References 430

18 Viral vaccines: concepts, principles, and bioprocesses 435
Isabel Maria Vicente Guedes de Carvalho Mello, Mateus Meneghesso da Conceição, Soraia Attie Calil Jorge, Pedro Estilita Cruz, Paula Maria Marques Alves, Manuel José Teixeira Carrondo, and Carlos Augusto Pereira
18.1 Introduction 435
18.2 Viral replication 436
18.2.1 Adsorption 437
18.2.2 Internalizing and unwrapping the viral particle 437
18.2.3 Structure and organization of viral genomes 437
18.2.4 Production and maturation of viral particles 442
18.3 Production of viral particles by cell culture 442
18.4 Strategies for the production of virus-like particles 447
18.4.1 Advantages of VLPs 448
18.4.2 VLP production technology 448
18.4.3 VLP composition 449
18.4.4 VLP production processes 450
18.5 Development of viruses for DNA vaccines 451
18.6 Perspectives for the evolution of viral vaccine production 452
References 455

19 Bioinsecticides 459
Márcia Regina da Silva Pedrini and Ronaldo Zucatelli Mendonça
19.1 Introduction 459
19.2 Baculovirus as a bioinsecticide: mechanism of action 460
19.3 Animal cell cultures for baculovirus production 463
19.4 Effect of culture medium, cell line, and virus isolate on biopesticide production 463
19.5 Polyhedra virulence and characteristics 466
19.6 Production of viral mutants in cell culture 467
References 470

20 Cell therapies and stem cells 475
Hamilton da Silva Jr and Radovan Borojevic
20.1 Introduction 475
20.2 Primary material 476
20.2.1 Stem and mature cells 477
20.2.2 Tissue environment and specific niches 484
20.3 Applications 485
20.3.1 Bioexpansion and biostorage 485
20.3.2 Bioengineering 486

20.4 Conclusions and perspectives 487
References 487

21 Gene therapy 489
Célio Lopes Silva, Karla de Melo Lima, Sandra Aparecida dos Santos, and José Maciel Rodrigues Jr
21.1 Introduction 489
21.2 Gene therapy 489
21.3 Vectors used in gene therapy 491
21.3.1 Viral vectors 491
21.3.2 Synthetic vectors: plasmid DNA 493
21.4 Principles of gene therapy 497
21.4.1 Replacement or correction of a mutant gene 497
21.4.2 Introduction of a heterologous gene 498
21.4.3 Gene inactivation 498
21.5 Gene therapy and clinical studies 498
21.5.1 The first gene therapy product 501
21.6 Perspectives 502
References 502

Appendix 505
Case study: Mathematical modeling of the monoclonal antibody anti-TNP (trinitrophenyl) 505

Index 507

Contributors

Paula Marques Alves, Instituto de Biologia Experimental e Tecnológica (IBET-ITQB), Portugal

Paola Amable, Cell Culture Engineering Laboratory, PEQ/COPPE, Federal University of Rio de Janeiro, Brazil

Elisabeth de Fatima Pires Augusto, Institute of Technological Research of the State of São Paulo, Brazil

Manuel Filgueira Barral, Fundação Santo André, Brazil

Mariela Bollati-Fogolín, Cell Biology Unit, Institut Pasteur de Montevideo, Uruguay

Radovan Borojevic, Institute for Biomedical Sciences, Federal University of Rio de Janeiro, Brazil

Sonia Maria Alves Bueno, Department of Biotechnological Processes, School of Chemical Engineering, State University of Campinas, Brazil

Michael Butler, Department of Microbiology, University of Manitoba, Canada

Manuel José Teixeira Carrondo, Instituto de Biologia Experimental e Tecnológica (IBET-ITQB), Portugal

Leda dos Reis Castilho, Cell Culture Engineering Laboratory, PEQ/COPPE, Federal University of Rio de Janeiro, Brazil

Mateus Meneghesso da Conceição, Laboratory of Viral Immunology, Butantan Institute, Brazil

Ernesto Chico, Center of Molecular Immunology, Cuba

Marcelo A. Comini, Centre of Biochemistry, Heidelberg University, Germany

Pedro Cruz, Instituto de Biologia Experimental e Tecnológica (IBET-ITQB), Portugal

Marina Etcheverrigaray, Facultad de Bioquímica y Ciencias Biológicas, Universidad Nacional del Litoral, Argentina

Alvio Figueredo, Center of Molecular Immunology, Cuba

Adriana Lages Lima Galesi, Department of Biotechnological Processes, School of Chemical Engineering, State University of Campinas, Brazil

Soraia Attie Calil Jorge, Laboratory of Viral Immunology, Butantan Institute, Brazil

Ricardo Kratje, Facultad de Bioquímica y Ciencias Biológicas, Universidad Nacional del Litoral, Argentina

Patrícia Léo, Institute of Technological Research of the State of São Paulo, Brazil

Karla de Melo Lima, Nanocore Biotecnologia Ltda, Brazil

Leila Costa Duarte Longa, Fundação Oswaldo Cruz, Brazil

Ricardo de Andrade Medronho, Chemical Engineering Department, School of Chemistry, Federal University of Rio de Janeiro, Brazil

Maria Candida Maia Mellado, Instituto de Biologia Experimental e Tecnológica (IBET-ITQB), Portugal

Isabel Maria Vicente Guedes de Carvalho Mello, Laboratory of Viral Immunology, Butantan Institute, Brazil

Ronaldo Zucatelli Mendonça, Laboratory of Viral Immunology, Butantan Institute, Brazil

Ângela Maria Moraes, Department of Biotechnological Processes, School of Chemical Engineering, State University of Campinas, Brazil

Ana Maria Moro, Laboratory for Biopharmaceuticals in Animal Cells, Butantan Institute, Brazil

Ana Cristina Almeida Müller, Carminatti Schulz Propriedade Intelectual, Brazil

Marcia Regina da Silva Pedrini, Federal University of Rio Grande do Norte, Brazil

Maíra Peixoto Pellegrini, Cell Culture Engineering Laboratory, PEQ/COPPE, Federal University of Rio de Janeiro, Brazil

Carlos Augusto Pereira, Laboratory of Viral Immunology, Butantan Institute, Brazil

Rosane Aparecida Moniz Piccoli, Institute of Technological Research of the State of São Paulo, Brazil

Rodrigo Coelho Ventura Pinto, Cell Culture Engineering Laboratory, PEQ/COPPE, Federal University of Rio de Janeiro, Brazil

Maria Teresa Alves Rodrigues, Laboratory of Biopharmaceuticals in Animal Cells, Butantan Institute, Brazil

José Maciel Rodrigues Jr, Nanocore Biotecnologia Ltda, Brazil

Gryssell Rodríguez, Center of Molecular Immunology, Cuba

Sandra Aparecida dos Santos, Nanocore Biotecnologia Ltda, Brazil

Célio Lopes Silva, University of São Paulo, Brazil

Hamilton da Silva Jr, Institute of Biomedical Sciences, Federal University of Rio de Janeiro, Brazil

Claudio Alberto Torres Suazo, Department of Chemical Engineering, Federal University of São Carlos, Brazil

Wirla Maria S. C. Tamashiro, Institute of Biology, State University of Campinas, Brazil

Aldo Tonso, Department of Chemical Engineering, University of São Paulo, Brazil

Abbreviations and Symbols List

Chapter 1

ADA	adenosine deaminase deficiency
BEVS	baculovirus expression vector system
BHK	baby hamster kidney cell line
CHO	Chinese hamster ovary cell line
ELISA	enzyme linked immuno sorbent assay
mAb	monoclonal antibodies
MDCK	Madin-Darby canine kidney epithelial cells
MMR	measles, mumps, rubella
SCID	severe combined immunodeficiency
tPA	plasminogen activator
VLP	virus-like particle

Chapter 2

ATP	adenosine triphosphate
EDTA	ethylenediaminetetraacetic acid
DMSO	Dimethylsulfoxide
BHK	baby hamster kidney cell line
CHO	Chinese hamster ovary cell line
HEPES	N-(2-hydroxyethyl)piperazine-N′-(2-ethanesulphonic acid)
mAbs	monoclonal antibodies
μ	specific cell growth rate
μ_{max}	maximum specific cell growth rate
MTT	(3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H tetrazolium bromide)
PEG	polyethylene glycol
RNA	ribonucleic acid
t	cell culture time
t_d	cell doubling time
WHO	World Health Organization
X	cell concentration

Chapter 3

β-gal	beta-galactosidase
AAV	adeno-associated virus
AP	alkaline phosphatase
ARE	elements rich in adenosine and uridine nucleotides (AU-rich elements)
BHK	baby hamster kidney cells
bp	base pairs
BPV	bovine papilloma virus
CAT	chloramphenicol acetyl transferase
cDNA	complementary deoxyribonucleic acid
CHO	Chinese hamster ovary cell line
CMV	cytomegalovirus

COS	African green monkey kidney cells (CV1 cells transformed with a defective mutant of SV40)
Cre	recombinase (cyclization recombination)
CV1	African green monkey kidney cells
DEAE-dextran	diethylaminoethyl-dextran
DHFR	dihydrofolate reductase
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DOPE	dioleoylphosphatidyl ethanolamine
DOSPA	2,3-dioleoyloxy-*N*-[2(sperminecarbox-amido)ethyl]-*N*,*N*-dimethyl-1-propane-aminium-trifluoracetate
DOTMA	*N*-[2,3-(dioleyloxy) propyl]-*N*,*N*,*N*-trimethyl ammonium chloride
E. coli	*Escherichia coli*
E	erythromycine operon/repressor
EBV	Epstein-Barr virus
ECFP	enhanced cyan fluorescence protein
ECV	extracellular viral particles
EGFP	enhanced green fluorescence protein
ELISA	enzyme-linked immunosorbent assay
EYFP	enhanced yellow fluorescence protein
FACS	fluorescence-activated cell sorter
FdG	fluorescein di-β-D-galactopyranoside
FH2	dihydrofolate
FH4	tetrahydrofolate
FK506	human immunophilins
FLP	native recombinase isolated from the 2μm plasmid from *Saccharomyces cerevisiae*
FRAP	rapamycin-induced immunophilins
FRT	FLP recombination target
G418	geneticin
gDNA	genomic DNA
GFP	green fluorescence protein
GS	glutamine synthetase
GST	glutathione S-transferase
HAT	hypoxanthine aminopterin thymidine
HBK	hybrid cell line generated from the fusion of Burkitt lymphoma and HEK-293 cells
HEK-293	human embryonic kidney cells
hGH	human growth hormone
High-Five	TM BTI-TN-5B1-4 (cell line derived from the insect *Trichoplusia ni*)
hsp70	heat shock protein 70
HSV	herpes simplex virus
IPTG	isopropyl 1-thio-ß-D-galactopyranoside
IRES	internal ribosomal entry site
kb	kilobases
Lac	lactose operon/repressor
LCR	locus control region
LoxP	locus of crossover of P1
LUC	luciferase isolated from firefly
MCS	multiple cloning site

mRNA	messenger RNA
MSX	methionine sulfoximine
MTX	methotrexate
NSONS0	murine myeloma cell line
ONPG	o-nitrophenyl-β-D-galactoside
ORF	open reading frame
OV	occluded viral particles
pCMV	cytomegalovirus promoter
PCR	polymerase chain reaction
PEI	polyethyleneimine
PIP	pristinamycine
polyA	polyadenylation signal
pSV40	early promoter of SV40 virus
RIA	radioimmuno assay
RNA	ribonucleic acid
RU486	mifepristone
SAR	scaffold attached region
SEAP	secreted alkaline phosphatase
Sf-21	cell line originated from the ovary of the insect *Spodoptera frugiperda*
Sf-9	clone derived from the Sf-21 cell line
Sp2/0	mouse myeloma cell line
SV40	simian virus 40
Tet	tetracycline operon/repressor
TEV	catalytic domain of the nuclear inclusion a (NIa) from the tobacco etch virus
TK	thymidine kinase
Tn-368	cell line originated from the ovary of the insect *Trichoplusia ni*
Tn7	transposon 7
tPA	tissue plasminogen activator
tRNA	transfer ribonucleic acid
UTR	untranslated regions
X-gal	5-bromo-4-chloro-3-indolyl-ß-D-galactoside
YACs	yeast artificial chromosomes

Chapter 4

Acetyl-CoA	acetyl-coenzyme A
ADP	adenosine diphosphate
Ala	alanine
AMP	adenosine monophosphate
ATP	adenosine triphosphate
BHK	baby hamster kidney cell line
CC9C10	hybridoma cell line
CHO	Chinese hamster ovary cell line
CHSE	Chinook salmon embryo cell line
CMP-NANA	cytosine monophosphate-N-acetylneuraminic acid
CoQ	coenzyme Q
$CoQH_2$	dihydroubiquinone
DNA	deoxyribonucleic acid

EPO	erythropoietin
FAD	flavin adenine dinucleotide
$FADH_2$	1,5-dihydro- flavin adenine dinucleotide
Fruc 6-P	fructose-6-phosphate
GDP	guanosine 5′-diphosphate
Glc-6-P	glucose-6-phosphate
GlcN 6 P	glucosamine-6-phosphate
GlcNAc-1-P	N-acetylglucosamine-1-phosphate
GlcNAc-6-P	N-acetylglucosamine-6-phosphate
Gln	glutamine
Gly	glycine
GP1	immunoadhesin glycoprotein
GTP	guanosine 5′-triphosphate
HL-60	human promyelocytic leukaemic cell line
IgG	immunoglobulin G
IMDM	Iscove′s Modified Dulbecco′s Medium
LLC-PK(1)	porcine kidney cell line
mAb	monoclonal antibody
ManNAc6P	mannose-N-acetyl-6-phosphate
MDCK	Madin-Darby canine kidney cell line
MGH-U1	human bladder cancer cell line
MN12	mouse-mouse hybridoma cell line
NAD^+	nicotinamide adenine dinucleotide
NADH	reduced NAD
NADP	nicotinamide adenine dinucleotide phosphate
NADPH	reduced NADP
NANA	N-acetylneuraminic acid
NeuNAc	N-acetylneuraminic acid
OK	opossum kidney cell line
OMP	orotidine monophosphate
P	phosphate
P/O ratio	molar ratio of ATP synthesis and oxygen consumption
PER.C6	human retina-derived cell line
PP	pyrophosphate
PPC	pentose phosphate cycle
PQXB1/2	murine B-lymphocyte hybridoma cell line
RNA	ribonucleic acid
S I	sugar nucleoside transport system I
S II	sugar nucleoside transport system II
Sf9	insect cell line (*Spodoptera frugiperda*)
SP2/0	mouse myeloma cell line
SP2/0-Ag14	mouse myeloma cell line
TCA	tricarboxylic acid cycle
TNFR-IgG	tumor necrosis factor receptor-IgG
tPA	tissue plasminogen activator
UDP	uridine diphosphate
UDP	uridine diphosphate
UDP-GalNAc	UDP-N-acetylgalactosamine
UDP-GlcNAC	UDP-N-acetylglucosamine
UDP-NANA	uridine diphosphate N-acetylneuraminic acid

UDP-N-GNAc	UDP-N-acetyl-hexosamine
UMP	uridine monophosphate
UTP	uridine triphosphate
VO 203	hybridoma cell line
WI-38	Wistar Institute-38; human embryonic lung tissue cell line

Chapter 5

bFGF	basic fibroblast growth factor
ECGF	endothelial growth factor
ECM	extracellular matrix
EGF	epidermal growth factor
FDA	food and drug administration
FGF	fibroblast growth factor
IGF-1	insulin-like growth factor-1
IGF-2	insulin-like growth factor-2
IL-1	interleukin-1
IL-6	interleukin-6
NGF	nerve growth factor
PDGF	platelet-derived growth factor
PW	purified water
TGF	transforming growth factor
WFI	water for injection

Chapter 6

AGE	advanced glycation end products
BHK	baby hamster kidney cell line
CHO	Chinese hamster ovary cell line
DO	dissolved oxygen
ER	endoplasmic reticulum
FVIII	blood coagulation factor eight
FX	blood coagulation factor ten
G2	digalactosylated glycans
Gla	gamma-carboxyglutamic acid
GlcNAc	N-acetyl glucosamine
HEK	human embryonic kidney cells
HIF	hypoxia-inducible factor
IgG	immunoglobulin
OST	oligosaccharyltransferase enzyme
t-PA	tissue-type plasminogen activator
Xyl	xylose

Chapter 7

14-3-3	cytosolic protein that binds to phosphorylated Bad, inactivating it
A-1	anti-apoptotic member of Bcl-2 family
AIF	apoptosis-inducing factor
Akt	serine/threonine kinase
ALG-2	apoptosis-linked gene-2

ANT	adenine nucleotide translocator
AO	acridine orange
Apaf-1	apoptosis protease activating factor-1
Apo-I	apoptosis antigen-I (also known as Fas and CD95, a member of the TNF receptor family)
ATP	adenosine triphosphate
Bad	Bcl-X_L/Bcl-2 associated death promoter, pro-apoptotic member of Bcl-2 family
Bak	Bcl-2 antagonist/killer, pro-apoptotic member of Bcl-2 family
Bax	Bcl-2 associated x protein, pro-apoptotic member of Bcl-2 family
Bcl-2	B-cell lymphoma 2, anti-apoptotic member of Bcl-2 family
Bcl-w	anti-apoptotic member of Bcl-2 family
Bcl-X_L	long form of Bcl-X, anti-apoptotic member of Bcl-2 family
Bcl-X_S	short form of Bcl-X, pro-apoptotic member of Bcl-2 family
BH1	Bcl-2 homolog domain 1
BH2	Bcl-2 homolog domain 2
BH3	Bcl-2 homolog domain 3
BH4	Bcl-2 homolog domain 4
BHK	baby hamster kidney cell line
BHRF1	Epstein-Barr virus Bcl-2 homolog
Bid	BH-3 interacting domain death agonist, pro-apoptotic member of Bcl-2 family
Bik	Bcl-2 interacting killer, pro-apoptotic member of Bcl-2 family
Bim	Bcl-2 interacting mediator of cell death, pro-apoptotic member of Bcl-2 family
BIR	baculovirus IAP repeat
Bmf	Bcl-2 modifying factor, pro-apoptotic member of Bcl-2 family
Boo	Bcl-2 homolog of ovary, anti-apoptotic member of Bcl-2 family
CAD	caspase-activated DNAse
CARD	caspase recruitment domain
CDK	cyclin dependent kinase
CHO	Chinese hamster ovary cell line
CHOP	C/EBP-homologous protein
cIAP1	cellular inhibitor of apoptosis protein 1
cIAP2	cellular inhibitor of apoptosis protein 2
c-jun	cellular homolog of v-Jun avian sarcoma virus 17 oncogene
c-myc	cellular homolog of the oncogene v-myc of avian myelocytomatosis virus strain 29
CO_2	carbon dioxide
COS	simian fibroblasts (CV-1 cells) transformed by SV40, deficient in the origin of replication region
CrmA	cytokine response modifier A
DAPI	4′,6′-diamidino-2-phenylindole (a fluorescent dye)
dATP	deoxy-adenosine triphosphate
DD	death domain
DED	death effector domain
DFF40	DNA fragmentation factor of 40 kDa
DIABLO	direct inhibition of apoptosis protein IAP binding protein with low pI
DISC	death inducing signaling complex
DNA	deoxyribonucleic acid

E1B-19kDa	adenovirus Bcl-2 homologue
EB	ethidium bromide
FADD	Fas associated death domain
FAK	focal adhesion kinase
Fas	cell surface receptor, member of the TNF receptor family that promotes apoptosis (also known as CD95 and APO-1)
FasL	Fas ligand
FITC	fluorescein isothiocyanate
G_0	cell cycle Gap 0 phase
G_1	cell cycle Gap 1 phase
G_2	cell cycle Gap 2 phase
GADD153	growth arrest and DNA damage-inducible gene 153
HEK 293	human embryonic kidney cell line
HeLa	human cervical adenocarcinoma cell line
HMW-DNA	high molar mass DNA
HRK	harakiri, pro-apoptotic member of Bcl-2 family
HSP	heat shock proteins
IAP	inhibitor of apoptosis proteins
ICAD	inhibitor of caspase-activated DNAse
IGF-1	insulin-like growth factor 1
JNK	c-Jun N-terminal kinase
KSBcl-2	Kaposi's sarcoma-associated herpesvirus Bcl-2 homolog
LMW-DNA	low molar mass DNA
M	cell cycle mitosis phase
Mcl-1	myeloid cell leukemia 1, anti-apoptotic member of Bcl-2 family
M-CSF	macrophage colony stimulating factor
MEKK1	Map/Erk kinase kinase 1
NBD	nucleotide-binding domain
NEC	necrotic cell
NFκB	nuclear factor of immunoglobulin kappa locus in B cells
NGF	nerve growth factor
Noxa	pro-apoptotic member of Bcl-2 family
NS0	myeloma cell line
NVA	non-viable apoptotic cell
Omi/HtrA2	high temperature requirement protein A2
p15	15 kDa protein, truncated Bid
p53	53 kDa protein, tumor suppressor gene product that promotes apoptosis and is involved in the cell cycle control
PAK2	p21-activated kinase 2
PARP	poly (ADP ribose) polymerase
PLK	polo-like kinases
PTP	permeability transition pore
Puma	p53 upregulated modulator of apoptosis, pro-apoptotic member of Bcl-2 family
RNA	ribonucleic acid
S	cell cycle synthesis phase
SAPK	stress-activated protein kinase
Sf9	insect cell line derived from *Spodoptera frugiperda*
Smac	second mitochondrial activator of caspases
TB/C3	hybridoma cell line

t_d	doubling time
TNF	tumor necrosis factor
TNF-R1	tumor necrosis factor receptor 1
TRADD	TNF receptor I associated death domain
TRAF2	TNF receptor associated factor 2
TRAIL-R1	TNF-related apoptosis-inducing ligand receptor 1
TRAIL-R2	TNF-related apoptosis-inducing ligand receptor 2
TUNEL	nuclear terminal deoxynucleotidyl transferase-mediated dUTP nick-end-labeling
UPR	unfolded protein response
VA	viable apoptotic cell
VDAC	voltage-dependent anion channel
VNA	viable nonapoptotic cell
WD-40 repeats	repeated sequence of approximately 40 amino acids, usually tryptophan (W) and aspartate (D) residues
x-dUTP	deoxyuridine triphosfatase; x = biotin, DIG or fluorescein
XIAP	X-linked inhibitor of apoptosis protein, member of IAP family that inhibits apoptosis
$\Delta\psi m$	inner mitochondrial membrane potential

Chapter 8

In the definitions below, M, L and T are basic units of mass, length and time, respectively.

D	specific feed rate (T^{-1})
D_{Per}	specific perfusion rate (T^{-1})
GLC	glucose concentration ($M \cdot L^{-3}$)
GLC*	glucose concentration under substrate limitation ($M \cdot L^{-3}$)
GLC_{thres}	threshold glucose concentration ($M \cdot L^{-3}$)
GLN	glutamine concentration ($M \cdot L^{-3}$)
GLN*	glutamine concentration under substrate limitation ($M \cdot L^{-3}$)
K	first order constant for glutamine decomposition (T^{-1})
k_d	specific cell death rate (T^{-1})
$k_{d,max}$	maximum specific cell death rate (T^{-1})
$k_{d,min}$	minimum specific cell death rate (T^{-1})
k_{GLC}	glucose limitation constant to growth ($M \cdot L^{-3}$)
k_{GLC}^{d}	glucose limitation constant to death ($M \cdot L^{-3}$)
k_{GLC}^{LAC}	glucose limitation constant for lactate synthesis ($M \cdot L^{-3}$)
k_{GLN}	glutamine limitation constant to growth ($M \cdot L^{-3}$)
k_{GLN}^{MAb}	glutamine limitation constant for monoclonal synthesis ($M \cdot L^{-3}$)
k_{GLN}^{LAC}	glutamine limitation constant for lactate synthesis ($M \cdot L^{-3}$)
$k_{GLN}^{NH_3}$	glutamine limitation constant for ammonia synthesis ($M \cdot L^{-3}$)
$k_{i,GLC}^{d}$	glucose inhibition constant for death ($M \cdot L^{-3}$)
$k_{i,GLC}^{MAb}$	glucose inhibition constant for monoclonal synthesis ($M \cdot L^{-3}$)
$k_{i,GLN}^{d}$	glutamine inhibition constant for death ($M \cdot L^{-3}$)
$k_{i,LAC}$	lactate inhibition constant for growth ($M \cdot L^{-3}$)
k_{i,NH_3}	ammonia inhibition constant for growth ($M \cdot L^{-3}$)

k_{i,NH_4^+}	ammonium inhibition constant for growth ($M \cdot L^{-3}$)
$k_{i,P}$	product inhibition constant for growth ($M \cdot L^{-3}$)
$k_{i,P}^{P}$	product inhibition constant for product synthesis ($M \cdot L^{-3}$)
$k_{i,S}$	substrate inhibition constant for growth ($M \cdot L^{-3}$)
k_{LAC}^{d}	lactate limitation constant for death ($M \cdot L^{-3}$)
$k_{NH_3}^{d}$	ammonia limitation constant for death ($M \cdot L^{-3}$)
$k_{NH^+_4}^{d}$	ammonium limitation constant for death ($M \cdot L^{-3}$)
k_S	substrate limitation constant for growth ($M \cdot L^{-3}$)
k_S^P	substrate limitation constant for product synthesis ($M \cdot L^{-3}$)
k_S^S	substrate limitation constant for substrate uptake ($M \cdot L^{-3}$)
LAC	lactate concentration ($M \cdot L^{-3}$)
MAb	monoclonal antibody concentration ($M \cdot L^{-3}$)
m_S	specific substrate uptake rate for maintenance, or maintenance coefficient ($M \cdot M^{-1} \cdot T^{-1}$ or $M \cdot Cel^{-1} \cdot T^{-1}$)
NH_3	ammonia concentration ($M \cdot L^{-3}$)
NH_4^+	ammonium concentration ($M \cdot L^{-3}$)
OF	objective function
P	product concentration ($M \cdot L^{-3}$)
r_P	product synthesis rate ($M \cdot L^{-3} \cdot T^{-1}$)
r_S	substrate consumption rate ($M \cdot L^{-3} \cdot T^{-1}$)
r_X	cell growth rate ($M \cdot L^{-3} \cdot T^{-1}$ or $Cel \cdot L^{-3} \cdot T^{-1}$)
S	substrate concentration ($M \cdot L^{-3}$)
S*	substrate concentration under substrate limitation ($M \cdot L^{-3}$)
t	time (T)
X	cell concentration ($M \cdot L^{-3}$or $Cel \cdot L^{-3}$)
X_d	dead cell concentration ($M \cdot L^{-3}$or $Cel \cdot L^{-3}$)
X_t	total cell concentration ($M \cdot L^{-3}$or $Cel \cdot L^{-3}$)
X_V	viable cell concentration ($M \cdot L^{-3}$or $Cel \cdot L^{-3}$)
$Y_{LAC/GLC}$	glucose-to-lactate yield coefficient ($M \cdot M^{-1}$)
$Y_{NH_4^+/GLN}$	glutamine-to-ammonium yield coefficient ($M \cdot M^{-1}$)
$Y_{P/S}$	substrate-to-product yield coefficient (or factor) ($M \cdot M^{-1}$)
$Y_{X/S}$	substrate-to-cells yield coefficient (or factor) ($M \cdot M^{-1}$ or $Cel \cdot M^{-1}$)
$Y_{X/S}^{max}$	maximum substrate-to-cells yield coefficient (or factor) ($M \cdot M^{-1}$ or $Cel \cdot M^{-1}$)
$Y_{X_V/GLC}$	glucose-to-viable cells yield coefficient ($M \cdot M^{-1}$ or $Cel \cdot M^{-1}$)
$Y_{X_V/GLN}$	glutamine-to-viable cells yield coefficient ($M \cdot M^{-1}$ or $Cel \cdot M^{-1}$)
α	constant for growth-associated production ($M \cdot M^{-1}$ or $M \cdot Cel^{-1}$)
β	constant for non-growth-associated production ($M \cdot M^{-1} \cdot T^{-1}$ or $M \cdot Cel^{-1} \cdot T^{-1}$)
Δi	variation of element i in the time interval Δt ($M \cdot L^{-3}$ or $Cel \cdot L^{-3}$)
Δt	time interval (T)
μ_{LAC}	specific lactate synthesis rate ($M \cdot M^{-1} \cdot T^{-1}$ or $M \cdot Cel^{-1} \cdot T^{-1}$)
μ_{MAb}	specific monoclonal synthesis rate ($M \cdot M^{-1} \cdot T^{-1}$ or $M \cdot Cel^{-1} \cdot T^{-1}$)
μ_{NH3}	specific ammonia synthesis rate ($M \cdot M^{-1} \cdot T^{-1}$ or $M \cdot Cel^{-1} \cdot T^{-1}$)
μ_P	specific product synthesis rate ($M \cdot M^{-1} \cdot T^{-1}$ or $M \cdot Cel^{-1} \cdot T^{-1}$)
$\mu_{P,max}$	maximum specific product synthesis rate ($M \cdot M^{-1} \cdot T^{-1}$ or $M \cdot Cel^{-1} \cdot T^{-1}$)

μ_S	specific substrate consumption rate ($M \cdot M^{-1} \cdot T^{-1}$ or $M \cdot Cel^{-1} \cdot T^{-1}$)
μ_S*	specific substrate uptake rate under substrate limitation conditions ($M \cdot M^{-1} \cdot T^{-1}$ or $M \cdot Cel^{-1} \cdot T^{-1}$)
μ_X	specific cell growth rate (T^{-1})
$\mu_{X,max}$	maximum specific cell growth rate (T^{-1})
$\mu_{X,min}$	minimum specific cell growth rate (T^{-1})
μ_X^{ap}	apparent specific growth rate (T^{-1})

Other parameters . . . b_1, b_2, b_3, C_1, C_2, d_0, d_1, F_1, F_2, h_1, h_2, K, $k_{GLC}^{LAC\#}$, $k_{GLN}^{NH_3\#}$, k'_{i,NH_3}, $k_{i,GLC}^{MAb'}$, $k_{LAC}^{d\#}$, $k_{NH_3}^{d\#}$, k'_S, $k_S^{S\#}$, k_μ, P_m, P'_m, α_0, α_1, α_2, α_3, α_4, β_0, β_1, β_2, β_m, β*, δ, λ, $\Delta\mu_S^{max}$, $\Delta\mu_P^{max}$.

Chapter 9

The units defined for the variables listed below are given in the MLT unit system, except in cases where cell concentration (given as cell number) is involved. M represents mass, L length and T time.

a	interfacial gas-liquid area per volume of bioreactor ($L^2\ L^{-3}$)
CHO	Chinese hamster ovary cell line
D	dilution rate (T^{-1})
$D_{wash\ out}$	dilution rate at wash-out conditions (T^{-1})
DO*	equilibrium dissolved oxygen concentration ($M\ L^{-3}$)
DO	dissolved oxygen concentration ($M\ L^{-3}$)
ECS	extracapillary space
ICS	intracapillary space
F	bioreactor volumetric feed flow rate ($L^3\ T^{-1}$)
H_{O2}	Henry's constant ($T^2\ L^{-2}$)
k_L	global oxygen transfer coefficient ($L^3\ L^{-2}\ T^{-1}$)
k_La	volumetric oxygen transfer coefficient (T^{-1})
NS0	myeloma cell line
P	product concentration ($M\ L^{-3}$)
P_A	annual production obtained in a bioreactor (M)
P_{acc}	amount of product accumulated throughout a bioreactor run (M)
P_{O2}	partial oxygen pressure ($M\ L^{-1}\ T^{-2}$)
P_V	volumetric productivity of a bioreactor ($M\ L^{-3}\ T^{-1}$)
q_{O2}	specific oxygen consumption rate ($M\ cells^{-1}\ T^{-1}$)
q_P	specific product formation rate ($M\ cells^{-1}\ T^{-1}$)
q_S	specific substrate consumption rate ($M\ cells^{-1}\ T^{-1}$)
r_{GLC}	glucose consumption rate ($M\ T^{-1}$)
r_{O2}	oxygen consumption rate ($M\ T^{-1}$)
S	substrate concentration ($M\ L^{-3}$)
S_A	substrate concentration in the bioreactor feed ($M\ L^{-3}$)
t	culture time (T)
t_{growth}	time needed for cells to grow up to the concentration desired for the process (T)
t_{work}	number of working days per year (T)
t_{prep}	time required for the preparation of the bioreactor (includes cleaning and sterilization) (T)
t_{proc}	process time (T)
t_{prod}	duration of the production phase (T)

V	bioreactor working volume (L^3)
$X_{V,PERFUSATE}$	viable cell concentration in the perfusate stream (cel T^{-1})
Xv	viable cell concentration (cells L^{-3})
α	cell passage factor in perfusion processes (–)
ε	turbulence eddy dissipation ($L^2 T^{-3}$)
λ	Kolmogorov eddy length (microscale of turbulence) (L)
μ	specific growth rate (T^{-1})
μ_{MAX}	maximum specific growth rate (T^{-1})
ν	kinematic viscosity ($L^2 T^{-1}$)

Chapter 10

C	concentration of dissolved oxygen
C_S	concentration of dissolved oxygen in equilibrium with the gas phase
FACS	fluorescence activated cell sorter
FIA	flow injection analysis
k_La	volumetric oxygen transfer coefficient
pCO_2	partial pressure of dissolved carbon dioxide gas
P-I-D	proportional-integral-derivative controller
Q_{O2}	specific respiration rate

Chapter 11

In the definitions below, M, L, and T are basic units of mass, length, and time, respectively.

A_{min}	minimum settling area (L^2)
b	force field intensity ($L T^{-2}$)
c_v	volumetric concentration (dimensionless)
d	particle (or cell) diameter (L)
d_{50}	cut size (L)
d'_{50}	reduced cut size (L)
D	perfusion rate (T^{-1})
E	total separation efficiency (dimensionless)
E'	reduced total separation efficiency (dimensionless)
g	acceleration of gravity ($L T^{-2}$)
G	grade efficiency (dimensionless)
G'	reduced grade efficiency (dimensionless)
Q	feed flow rate ($L^3 T^{-1}$)
Q_C	concentrated stream (underflow) flow rate ($L^3 T^{-1}$)
Q_D	diluted stream (overflow) flow rate ($L^3 T^{-1}$)
r	radial position of the particle (L)
R	centrifuge radius (L)
R_f	flow ratio (dimensionless)
u	ascending velocity of the liquid in the settler ($L T^{-1}$)
V	bioreactor volume (L^3)
v_t	terminal velocity of the particle at low concentrations ($L T^{-1}$)
v_{tc}	terminal velocity of the particle at a concentration c_v ($L T^{-1}$)
x	particle size frequency in the feed suspension (dimensionless)
X	cell concentration, in number, in the feed suspension (L^{-3})
X_C	cell concentration, in number, in the concentrated suspension (L^{-3})

X_D	cell concentration, in number, in the diluted suspension (L^{-3})
X_d	concentration of cells with diameter *d*, in number, in the feed suspension (L^{-3})
X_{dC}	concentration of cells with diameter *d*, in number, in the concentrated suspension (L^{-3})
X_{dD}	concentration of cells with diameter *d*, in number, in the diluted suspension (L^{-3})
y	cumulative particle size distribution (undersize) of the feed suspension (dimensionless)
y_C	cumulative particle size distribution (undersize) of the concentrated suspension (dimensionless)
y_D	cumulative particle size distribution (undersize) of the diluted suspension (dimensionless)
z	cumulative particle size distribution (oversize) of the feed suspension (dimensionless)
μ	specific cell growth rate (T^{-1})
μ_{ap}	apparent specific cell growth rate (T^{-1})
μ_L	liquid viscosity ($M\,L^{-1}\,T^{-1}$)
ρ	density of the liquid ($M\,L^{-3}$)
ρs	density of the particles or cells ($M\,L^{-3}$)
ω	angular velocity (T^{-1})

Chapter 12

AE	Aminoethyl
C	Carboxy
C1	Methyl
C8	Octyl
C18	Octyldecyl
CM	Carboxymethyl
DEAE	Diethylaminoethyl
DS-2	*Drosophila melanogaster* schneider-2
DTE	1,4-dithioerythritol
DTT	1,4-dithiothreitol
EBA	expanded bed adsorption
EDTA	ethylenediaminetetraacetic acid
H	height of the expanded bed
H_0	height of the packed bed
HBsAg	hepatitis B virus surface antigen
HIC	hydrophobic interaction chromatography
K	partitioning coefficient
K_A	association constant
K_{av}	alternative distribution coefficient
K_d	distribution coefficient
K_D	dissociation constant
L	Ligand
MWCO	molecular weight cut-off
P	Protein
PEG	polyethylene glycol
pI	isoelectric point

PL	reversible complex ptotein-ligand
PVC	poly vinyl chloride
Q	quaternary amine
RPC	reverse phase chromatography
S	Sulfonate
SM	Sulfomethyl
SP	Sulfopropyl
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate – polyacrylamide gel electrophoresis
TEAE	Triethylaminoethyl
TAM	Trimethylaminomethyl
V_e	elution volume
V_o	interstitial volume of the porous matrix
V_s	total solvent volume within the pores
V_t	total column volume
x	solute concentration in the original solution
y	solute concentration in the extract

Chapter 13

CPE	Cytopathic effect
DNA	Deoxyribonucleic acid
GMP	Good manufacturing practise
Had	hemadsorption
HPCE	High performance capillary electrophoresis
HPLC	High performance liquid chromatograpy
HPSEC	High-performance size-exclusion chromatography
IEF	Isoelectric focusing
mAbs	Monoclonal antibodies
MCB	Master cell bank
mRNA	Messenger RNA
PCR	Polymerase chain reaction
pI	Isoelectric point
PMA	Production of monoclonal antibodies
PVDF	Polyvinylidene fluoride
rDNA	Recombinant DNA
RNA	Ribonucleic acid
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate – polyacrylamide gel electrophoresis
UV	Ultra violet
WCB	Working cell bank
WHO	World Health Organization

Chapter 14

ANVISA	Agência Nacional de Vigilância Sanitária (Brazilian National Agency for Sanitary Vigilance)
BL	biosafety level
BLA	Biologics License Application

BSE	bovine spongiform encephalopathy
CBER	Center for Biologics Evaluation and Research
CDER	Center for Drug Evaluation and Research
cGMP	current good manufacturing practices
CHO	Chinese hamster ovary cell line
CNS	Conselho Nacional de Saúde (Brazilian National Health Council)
CPMP	Committee for Proprietary Medicinal Products
CTNBio	Comissão Técnica Nacional de Biossegurança (Brazilian National Biosafety Technical Commission)
EC	European Commission
EMEA	European Medicines Agency
FDA	Food and Drug Administration
GCP	good clinical practices
GMO	genetically modified organisms
HAP	hamster antibody production
HEPA	high efficiency particulate air
HIV	human immunodeficiency virus
HTLV	human T-Cell leukemia virus
ICH	International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use
LSBL	large-scale biosafety level
MAP	mouse antibody production
MCB	master cell bank
NDA	new drug application
NIH	National Institutes of Health
PPC	post-production cells
PTC	points to consider
QA	quality assurance
QC	quality control
RAP	rat antibody production
WCB	working cell bank
WCP	well-characterized product
WHO	World Health Organization

Chapter 15

ADR	alternative dispute resolution
AUTM	Association of University Technology Managers
EPC	European Patent Convention
IDA	irnationally recognized depositary authority
IFN-alpha	Interferon-alpha
INPI	Brazilian National Institute of Industrial Property
mAb	Monoclonal antibody

Chapter 16

BLA	Biologics License Application
CG	chorionic gonadotrophin
CHO	Chinese hamster ovary cell line
CSF	colony-stimulating factor

EGF	epidermal growth factor
EMEA	European Medicines Agency
EPO	erythropoietin
FDA	Food and Drug Administration
FIX	blood coagulation factor IX
FSH	follicle-stimulating hormone
FVII	blood coagulation factor VII
FVIII	blood coagulation factor VIII
G-CSF	granulocyte colony-stimulation factor
GM-CSF	granulocyte-macrophage colony-stimulation factor
IFN	interferon
IGF	insulin-like growth factor
IgG	immunoglobulin G
IL	interleukin
IND	investigational new drug application
LH	luteinising hormone
mAb	monoclonal antibody
M-CSF	macrophage colony-stimulation factor
PDGF	platelet-derived growth factor
PEG	polyethylene glycol
TGF	transforming growth factor
TNF	tumor necrosis factor
TNKase	tenecteplase (commercial second-generation tPA)
tPA	tissue plasminogen activator
VEGF	vascular endothelial growth factor

Chapter 17

ATP	Adenosine triphosphate
BHK	baby hamster kidney cell line
CD	cluster of differentiation
cDNA	complementary DNA
CDR	Complementarity determining regions
CHO	Chinese hamster ovary cell line
CMC	carboximethylcellulose
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DO	dissolved oxygen
ELISA	enzyme linked immunosorbent assays
Fc	functional fragments
Fv	variable fragments
HACA	Human Anti-Chimeric Antibody
HAMA	Human Anti-Murine Antibody
HAT	aminopterin, hypoxanthine, and thymidine
HEK-93	human embryonic kidney cell line
HGPRT	hypoxanthine phosphoribosyl transferase
Ig	Immunoglobin
IgA	Immunoglobin class A
IgD	Immunoglobin class D

IgE	Immunoglobin class E
IgG	Immunoglobin class G
IgM	Immunoglobin class M
IL	Interleukin
K_La	Volumetric oxygen transfer coefficient
KLH	keyhole limpet haemocyanin
mAb	Monoclonal antibody
PEG	polyethylene glycol
S-S	Disulfide bridge
TK	thymidine kinase
tRNA	transcript of the RNA

Chapter 18

BTV	Blue tongue virus
cccDNA	Covalently closed circular DNA
CLPs	Core-like particles
CTL	Cytotoxic T-lymphocytes
dDNA	Double-strand DNA
dRNA	Double-strand RNA
HBsAg	hepatitis B virus surface antigen
HBV	Hepatitis B virus
mRNA	Messenger rNA
+mRNA	Positive strand messenger RNA
PPV	Porcine parvovirus
RNA-	Complementary negative RNA strand
RNA+	Genomic RNA
-sRNA	Negative single-strand RNA
+sRNA	Positive strand RNA
RNAs	Genomic RNA
ssRNA	Single-stranded RNA
MOI	Multiplicity of infection
TOI	Time of infection
VLPs	Virus-like particles
VP1	Viral protein 1
VP2	Viral protein 2
VP3	Viral protein 3
VP4	Viral protein 4
VP5	Viral protein 5
VP6	Viral protein 6
VP7	Viral protein 7

Chapter 19

AcMNPV	*Autographa californica* NPV
AfMNPV	*Anagrapha falcifera* nucleopoliedrovirus
BmMNPV	*Bombyx mori* NPV
BV	budded virus
DIP	Defectives Interfering Particles mutants
DNA	Deoxyribonucleic acid

EMBRAPA	Brazilian Agricultural Research Corporation
FBS	fetal bovine serum
FP	Few Polyedra mutants
GmMNPV	*Galleria mellonella* NPV
GMO	genetically modified organisms
GV	Granulovirus
HaSNPV	*Helicoverpa armigera* NPV
HzSNPV	*Helicoverpa zea* nucleopoliedrovirus
IEA	Institute of Agriculture
LdMNPV	*Lymantria dispar* nucleopoliedrovirus
MNPV	multiple NPV type
MOI	multiplicity of infection
NPV	Nucleopolyedrovirus
OB	occlusion body
ODV	occlusion-derived virus
OpMNPV	*Orgyia pseudotsugata* NPV
Sf9 and Sf21	*Spodoptera frugiperda* cell line
SfMNPV	*Spodoptera frugiperda* NPV
SNPV	simple NPV type
TN-368	*Trichoplusia ni* cell line
TnMNPV	*Trichoplusia ni* NPV

Chapter 20

3G5	membrane ganglioside related to pericytes identification
BMP-12R	receptor to bone morphogenetic protein isoform 12
Brachyury	transcription factor related to embryo mesoderm determination
CBF	core-binding factor; protein family involved in cell differentiation processes
CD3/cCD3	protein complex tyrosine kinase associated with T cell receptor (TCR); detected on cell surface or intracytoplasm (c)
CD4	TCR co-receptor; binds to MHC-II (major histocompatibility complex – class II)
CD7	transmembrane protein linked to PI-3 (phosphoinositol-3) kinase; cellular function not thoroughly clarified
CD8	TCR co-receptor; binds to MHC-I
CD10	metalloproteinase Zn-bound
CD11b	integrin α_M; associated with CD18 (integrin β_2); CD54 ligand; binds complement system iC3b and ECM (extracellular matrix) proteins; also known as Mac-1
CD11c	integrin α_X; associated with CD18; fibrinogen ligand; also known as Mac-3
CD13	aminopeptidase N (metalloproteinase Zn-bound)
CD14	LPS (lipopolysaccharide) receptor
CD16/32	FcγRII/III (receptor II/III to Fcγ)
CD19	B cell receptor co-receptor linked to PI-3 kinase; forms complex ligands with CD21 (C3b receptor) and CD81
CD25	IL-2 α chain receptor
CD29	β_1 integrin ; forms a complex with CD49/α-integrin
CD31	PECAM/platelet-endothelial cell adhesion molecule

CD33	gp67/glycoprotein 67 expressed by myelomonocytic progenitors; function not thoroughly yet defined
CD34	gp105-120; binds to CD62L (L-selectina); antigen essential to identify human stem cells; function not yet completely known
CD35	C1 and C4b complement protein system receptor
CD38	NAD glycohydrolase; expression related to cell proliferation increase
CD41	integrin/gpIIb α^{IIb} chain; works with CD61 (gpIIIa), binding to fibrinogen, vWF and trombospondin
CD44	H-CAM (Hermes cell adhesion molecule); binds to hialuronic acid and promotes mild leukocyte interaction
CD45	LCA/leukocyte common antigen; tyrosine phosphatase that reinforces T and B lymphocyte receptor signaling
CD45RA	isoform of CD45 containing the exon A; expressed on naïve T, B cells and monocyte subpopulations
CD49	α chain integrin; involved in cell interactions
CD54	ICAM-1 (intercellular adhesion molecule); this binds to CD11a-b/CD18 complex
CD56	NCAM (neural cell adhesion molecule); good marker for NK cells
CD60	9-O-acetyl GD3; surface ganglioside involved in receptor complexes during cell signaling
CD62L	L selectin; related to co-binding with CD34 and interactions to endothelia
CD64	FcγR1/receptor 1 to Fc gamma
CD66a	BGP (biliary glycoprotein); member of carcino-embryonary antigen (CEA) family
CD71	receptor to transferrin
CD73	ecto-5'-nucleotidase; involved on nucleotide dephosphorylation
CD81	TAPA-1 (TAPA-1 – target for antiproliferative antigen-1); associated with CD19 and CD21
CD87	plasminogen receptor activator
CD90	also known Thy-1; T-lymphocyte marker involved in cellular adhesion
CD100	semaphorin subtype; function not completely defined
CD104	β4 integrin chain; associated with CD49 and binds to laminin
CD105	endoglin; TGF-β co-factor
CD106	VCAM-1 (vascular cell adhesion molecule); this binds to VLA-4 (very late antigen)
CD110	trombospondin receptor
CD117	SCF (stromal or stem cell factor) receptor; mostly known as cKit
CD122	β-chain of IL-2receptor
CD123	α-chain of IL-3 receptor
CD125	α-chain of IL-5 receptor
CD127	α-chain of IL-7 receptor
CD135	FLT-3/Flk-2; also known as Flt-3L (fms-related tyrosine kinase ligand) receptor
CD144	VE-cadherin; adhesion molecule related to endothelial cells
CD146	MCAM (mesenchymal cell adhesion molecule); also known as MUC-18, S-endo or MEL-CAM
CD166	gp37; molecule involved in adhesion between lymphocytes and thymic epithelial cells

CD203c	ecto-enzyme ectonucleotide pyrophosphatase/phosphodiesterase 3 (E-NPP3); mostly expressed in basofiles
CD235a	glycophorin A; membrane protein expressed in red blood cells
Col	collagen
cTn	cardiac throponin
CXCR4	chemokine receptor CXC 4
EPO-R	receptor of erythropoietin
F4/80	membrane glycoprotein related to macrophage activity
FGF-R	fibroblast growth factor receptor
GM-1	membrane ganglioside related to nervous cells
GM-CSFR	granulocyte/monocyte - colony stimulation factor receptor
Gr-1	membrane antigen expressed mainly on granulocytes
HLA-DR	human leukocyte antigen – DR; analogous to mice MHC
HLA-II	HLA class II
IgE-R	immunoglobulin E receptor
IL-5R	receptor of IL-5
KDR	also known as flk-1 and VEGF-R2 (vascular/endothelial growth factor receptor 2)
Lin	abbreviation for lineage; related to hematopoietic cell lineages, namely: CD3/4/8/11b/19/56, Gr-1 and Ter 119 (mice) ou CD235a (human)
LPL	L lipoprotein
M-CSFR	monocyte - colony stimulation factor receptor
MHC	myosin heavy chain
MHC-II	MHC class II
MMCP	mouse mast cell protease
MMP	metalloproteinase
NG2	proteoglycan heparan-sulfate related to pericytes activity
PDGF	platelet-derived growth factor
PPAR	peroxisome proliferator-activated receptor gamma
RANK	receptor activator of NF-kappa β
Sca-1	stem cell antigen 1
SM22	smooth cell protein; structurally related to calponin, both actin and tropomyosin ligands
SMA	smooth muscle actin
SSEA-I	stage-specific embryonic antigen; characterized as a glycosphingolipid similar to Ii (MHC invariant chain)
Stro-1	stromal cell surface antigen, called stromal antigen 1
Ter-119	mice red blood cell marker
Tie-2	receptor tyrosine kinase related to endothelial cells activity
VEGF-R1	receptor 1 of VEGF
VEGF-R3	receptor 3 of VEGF
vWF	Von Willebrand factor. Protein factor involved in hemostasis

Chapter 21

DNA	deoxyribonucleic acid
RNA	ribonucleic acid
iRNA	interference RNA
AAV	adeno-associated virus
HSV	herpes simplex virus

Foreword

In vitro culture of animal cells originated towards the end of the nineteenth century as a collection of experimental procedures used to isolate and maintain viable cells from organs of diverse animals, at least for a few days. From these modest origins, and throughout a little more than 100 years, animal cell culture has evolved into a modern technology based on scientific and engineering principles. As a result of such developments, animal cell culture is nowadays used successfully for the production of vaccines and recombinant glycoproteins. In addition, applications of animal cell culture now underpin new and fascinating fields, such as organ transplants, cell and gene therapy, *in vitro* toxicology and physiology, tissue analogs, production of biopesticides, bioelectromechanical devices, and nanobiotechnology. Interestingly, of the more than 500 biopharmaceutical products that are being evaluated in clinical trials, about half are produced by animal cell culture. Such facts underline the importance of this technology. Furthermore, the products derived from animal cells have an annual market of more than ten thousand million dollars with impressive growth rates. Nonetheless, far more important than their economic value is the impact that such products have made on human health, increasing the quality of life.

The first therapeutic product generated by animal cells, tPA, was approved in 1986. In 1987, the first therapeutic monoclonal antibody, OKT3, was also approved, although this protein was obtained from the ascites fluid produced *in vivo*. It is notable that in merely two decades, close to 50 products produced by *in vitro* animal cell culture have now been approved. From these, approximately 60% are monoclonal antibodies. Such an achievement was the result, to a large extent, of the extraordinary advances that animal cell culture technology has experienced since the 1980s. Since then, cell and product concentrations have increased between one to two orders of magnitude, yielding bioprocesses that can routinely attain more than 50×10^6 cell/mL and 5 g/L of product. Likewise, due to advances in cell line selection and new expression systems, specific cell productivities above 100 pg/cell-d are now a reality. The problem of cell fragility, that drew the attention of researchers during the 1980s and 90s, was adequately overcome. During the last two decades, an important effort was focused on cell metabolism and physiology. Such understanding allowed, among other things, a better definition of culture media. Consequently, it was possible to eliminate undefined and undesirable components, such as fetal bovine serum, and chemically defined media became widely used. Knowledge of cell death processes resulted in novel culture schemes and molecular strategies to contend with this problem. With the improvement in analytical methods and comprehension of protein post-translational modifications, a considerable advancement was possible in understanding the relation between the bioprocess and the quality of the glycoproteins produced. All these developments permitted the practical, efficient, and robust culture of animal cells in bioreactors at scales larger than 10 m^3 for consistently producing safe and effective biopharmaceuticals.

In the present book, *Animal Cell Technology: From Biopharmaceuticals to Gene Therapy*, editors Drs Leda R. Castilho, Ângela M. Moraes, Elisabeth F. Pires Augusto and Michael Butler have integrated the background knowledge and developments of the last 20 years that have converted the culture of animal cells into a vigorous and important scientific field. Accordingly, basic themes are reviewed in the book, including cloning and expression

of heterologous proteins, culture media, metabolism, cell growth and death. Technological aspects are also examined, including the design, monitoring and control of bioreactors, and processes for separation of cells and products. Topics of major relevance, such as the quality control of products, regulatory issues and intellectual property, are included in several chapters. Finally, some of the most important products and applications of animal cell culture, including recombinant glycoproteins, monoclonal antibodies, viral vaccines, bioinsecticides, and gene and cell therapies, are reviewed in detail.

This book includes contributions of distinguished researchers from Canada and Ibero-America, in particular, from Brazil, Argentina, Cuba, Portugal and Uruguay. These authors impart a special character to the book, since it reflects the global impact that the discipline has acquired, and provides the vision and topics that are of interest to countries of this region. It also represents an additional effort, started in 2004 by Drs Leda R. Castilho and Ricardo Medronho during the ''first Latin-American Seminar on Cell Culture Technology'', to strengthen the relationship between Ibero-American technologists and scientists working in animal cell culture.

The reader of the present book will be able to recognize that there still exist major technological and scientific challenges, as well as promising opportunities, in the field of animal cell culture. It is possible to anticipate important changes in paradigms that will determine the future of the field. Twenty years after the first products were approved, many patents are now starting to expire. This has brought to a central stage the discussion of the concept of biogenerics, not only from a commercial perspective, but also from a technical and regulatory standpoint. Such a topic should be dealt with from a scientific approach and particularly supported by detailed analytical characterization of the molecules and their therapeutic efficacy, aspects in which there has been outstanding recent progress. Progress in this area will certainly contribute to defeat dogmas such as ''the process determines the product'', which in turn will open new possibilities for developing improved and novel bioprocesses that efficiently yield safe biopharmaceuticals at accessible prices to a larger number of patients. Advances in genomics and proteomics should contribute in bringing to the market an increasingly large number of products, derived from animal cell culture, for treating an increasing number of diseases. Again, new technological, medical and regulatory paradigms will most likely be seen with the advent of cell and gene therapies. Monoclonal antibodies, that are required in very large amounts, are finally fulfilling the therapeutic promises generated in the mid-70s, and with this, new manufacturing challenges can be foreseen. The race to develop bioprocesses that can yield concentrations above 10 g/L has started. It is likely that, by integrating the knowledge generated in the field during the last two decades, such a goal will be reached in a short time. A few examples that will contribute to reaching such a goal include novel operation and control systems, new culture media supplemented with hydrolyzed proteins from plant or yeast, and metabolic engineering of novel cell lines. New paradigms will also be established in the purification of biopharmaceuticals. Accordingly, it is likely that soon we will see the application of unit operations that are presently uncommon for the field, such as precipitation, crystallization, and extraction, whereas the importance of common but costly methods used nowadays, such as chromatography, will diminish in large-scale operations. This should bring a simplification and cost reduction of bioprocessing. On the other hand, regulatory requirements will most likely affect and define the future of animal cell culture. A clear example of this is the trend to use disposable equipment and instrumentation, which should represent a fertile area for innovation.

It is clear that animal cell culture will remain an exciting and highly dynamic field in the years to come, and that the products generated by this technology will benefit an increasing number of people. Accordingly, the book *Animal Cell Technology: From Biopharmaceu-*

ticals to Gene Therapy should be an important ally for technologists and scientists interested in the topic.

Octavio T. Ramírez
Instituto de Biotecnología, UNAM
Cuernavaca, México

Cover Photo Acknowledgement

Photo 1

Clumps of suspension-adapted CHO cells stained with ethidium bromide and acridine orange, observed under a fluorescence microscope. Photo taken by Dr. Rodrigo C. V. Pinto, Cell Culture Engnieering Laboratory, Federal University of Rio de Janeiro, Brazil.

Photo 2

Adherent CHO cells. Photo taken by Dr. Rodrigo C. V. Pinto, Cell Culture Engnieering Laboratory, Federal University of Rio de Janeiro, Brazil.

Photo 3

Laboratory-scale stirred-tank bioreactor for animal cell cultiavaion. Photo taken by Dr. Rodrigo C. V. Pinto, Cell Culture Engnieering Laboratory, Federal University of Rio de Janeiro, Brazil.

Photo 4

Inner surface of an industrial-scale stirred-tank bioreactor for animal cell cultivation. Photo taken by Dr. Ernesto Chico, Center of molecular Immunology, Cuba.

Introduction to animal cell technology

1

Paula Marques Alves, Manuel José Teixeira Carrondo, and Pedro Estilita Cruz

1.1 Landmarks in the culture of animal cells

Despite the dominance of animal cell culture in the production of biopharmaceuticals in recent times, this technology was not consolidated into standardized large-scale bioprocesses until the 1990s. Nevertheless, the first experience with animal cell culture can be traced back to the beginning of the 20th century. By the use of the hanging drop technique and frog heart lymph, Ross Harrison, at Yale, tried between 1906 and 1910 to elucidate how the nervous fiber is originated (Witkowski, 1979). He considered three hypotheses: (i) *in situ* formation from the nerve sheath; (ii) preformed protoplasmic bridges; or (iii) as a result of the nerve cell growth itself. When Harrison demonstrated the validity of the third hypothesis, he also confirmed the cell as the primary developing unit of multicellular organisms.

An early pioneer of cell culture was the French surgeon Alexis Carrel, who won the Nobel prize in Medicine in 1912 for his research at the Rockefeller Institute (Spier, 2000). Harrison was, above all, the inventor of analytical solutions, while Carrel, with his extensive clinical practice experience, sterility concerns, and capacity to develop appropriate culture media and culture flasks, created the change in the technological paradigm that led to the start up of animal cell technology. By careful manipulation, Carrel insured the maintenance of chicken embryo cells for several decades in culture.

Spier (2000) lists some essential differences between cells in an organism (*in vivo*) and cells in culture (*in vitro*), particularly the following;

(i) Tissues are three-dimensional, while cell cultures are of zero dimension (monodispersed in suspension culture) or two-dimensional (monolayer growth). However, some culture techniques exploit three-dimensional systems (Alves *et al.,* 1996; Powers *et al.,* 2002).
(ii) In tissues, cells are subject to tension and compression, but not when in culture, with the exception of artificial organs.
(iii) In tissues, lymphokines and chemokines vary in proportion and concentration to allow fluctuations of short (cardiac rhythm), medium (daily), and long duration (life cycle). However, in culture these parameters normally do not vary.
(iv) The mechanisms for cellular differentiation control in tissues and in culture are distinct.

The need to deal with some of these differences demands enormous efforts for the development of culture media (chemical environment) or shear, mixing, viscosity and bubbling conditions (physical environment), which should be optimized to result in an industrial process that can be validated. Requirements to avoid contamination have led to the formulation of serum-free media or even of protein-free, chemically defined media for the production of biopharmaceuticals (Griffiths, 1988).

The range of culture flasks and reactor types employed is quite wide, both for suspension and adherent cultures, from small Carrel's or Roux's flasks to roller bottles. Fixed- and fluidized-bed bioreactors, air-lift reactors and even stirred and aerated tanks with capacities up to 15 m^3 are common in large plants producing monoclonal antibodies (mAbs) for anticancer therapies (Adams and Weiner, 2005; Griffiths, 1988).

One of the main purposes of animal cell culture development was the search for viral vaccines, initiated during the Second World War (1939–1945), particularly for poliomyelitis. The names of Enders, Syverton, and Salk are undoubtedly associated with the production of the inactivated polio vaccine, approved in the USA in 1955 and produced on a large scale in primary monkey kidney cells. Later, after a dispute between Hilary Kaprowski and Albert Sabin, the attenuated vaccine against poliomyelitis was licensed in 1962. At the end of this period, at the Wistar Institute, Hayflick developed a cell line from embryonic tissue capable of replicating more than 50-fold before becoming senescent (Hayflick and Moorhead, 1961). The cell was diploid, easy to freeze and to reactivate and did not show any evidence of contamination by the viruses normally found in monkey primary kidney cells. This cell line (WI-38) turned into the basis for the production of human viral vaccines against poliomyelitis and MMR (measles, mumps, rubella), while other cell lines were evaluated for the production of veterinary vaccines, such as BHK (baby hamster kidney) in the case of the vaccine against foot-and-mouth disease.

After this period, there was an accelerated use of animal cells. Namalwa cells (Finter *et al*., 1991) were used to produce alpha-interferon by Wellcome in 1986. Vero cells (a cell line derived from monkey) were used for anti-rabies vaccine. Hybridomas were considered acceptable for mAb production and the use of genetically modified CHO (Chinese hamster ovary) cells was authorized for the production of tissue plasminogen activator (tPA) by Genentech in 1986.

Finally, three relevant aspects should be mentioned to clarify the scientific, technological, and industrial position of biopharmaceuticals and animal cells.

(i) Complex biopharmaceuticals, such as proteins, virus or virus-like particles (VLPs), among others, produced by cellular and/or recombinant technologies are characterized/defined by their own production processes. This means that analytical, biological and immunological characterization assays are usually not considered sufficient for product marketing, given the complexity of the molecules. Therefore, product licensing is based on the specific production process, which cannot be altered. Process changes may require new licensing proce-

dures, and this makes the introduction of biosimilars in the market more difficult.

(ii) Some of these proteins (for therapy) or VLPs (for vaccines such as hepatitis B) can be produced by yeast or even by *Escherichia coli* because of the limited requirement for post-translational modification (see Chapter 6). The total market value of biopharmaceuticals produced by *E. coli* or yeast was surpassed by those produced by animal cells only around 1996. In the last few years, market dominance in favor of animal cells has increased significantly.

(iii) Considering the complexity and instability of biopharmaceuticals, the production process from animal cells has to be designed, modeled, and optimized in an integrated form, taking into account the culture, extraction, and separation (Cruz *et al.*, 2002). This may be different from what is normal in the production of simple biological compounds such as antibiotics or vitamins.

The number of biological processes has increased tremendously in the last 15 years. This has resulted in high expectation for an improvement of quality of life and an increase in the volume of business related to products obtained from animal cell culture technology, with the broad potential use of these products for disease diagnostics, prevention, therapy, and cure. *Table 1.1* indicates some examples of approved therapeutic products obtained through animal cell culture.

1.2 Types of animal cell cultures

The methods developed for obtaining and maintaining primary cultures paved the way for animal cell technology. However, the huge growth and expansion of this technology was possible only because new cell types were established, namely diploid cells, hybridomas, and other continuous cell lines. Animal cells in culture can be classified, according to their origin

Table 1.1 Examples of approved products obtained through animal cell culture

Product	Protein	Use	Cell	Approval year
Avonex®	β Interferon	Multiple sclerosis	CHO	1996
BeneFix®	Factor IX	Hemophilia B	CHO	1997
Epogen®	Erythropoietin	Anemia	CHO	1989
Gonal f®	Follicle stimulating hormone	Female infertility	CHO	1995
Herceptin® / trastuzumab	mAb	Breast cancer	CHO	1998
Kogenate®	Factor VIII	Hemophilia A	BHK	1993
Simulect® / basiliximab	mAb	Acute transplanted kidney rejection	Murine myeloma	1998
Campath® / alemtuzumab	Humanized mAb	Leukemia	CHO	2001
Xolair® / omalizumab	Humanized mAb	Asthma	CHO	2003
Avastin® / bevacizumab	Humanized mAb	Colon or rectum carcinoma	CHO	2004

and biology, into primary cultures and cell lines (nomenclature most frequently employed in textbooks). However, depending on their applications, animal cells can also be grouped as follows.

(i) Cells producing proteins employed in the production of complex therapeutics, subunit vaccines, and diagnostic products, such as CHO, BHK, HEK-293, WI-38, MRC-5, SP2/0, NS0, and insect cells.
(ii) Cells producing viruses used in gene therapy and viral gene vaccines (for instance, Vero, HEK-293, and PER.C6® cells).
(iii) Normal cells, tumor cells, and stem cells used in research and development, specifically in the discovery of new products and for *in vitro* study and toxicology models (e.g. nerve cells, fibroblasts, Caco-2, MRC-5, and endothelial cells).
(iv) Human cells for subsequent use in cell therapy and regenerative medicine (e.g. embryonic and adult stem cells).

Primary cells are isolated directly from organs or tissues. Primary cells are normally heterogeneous and better represent the tissue from which they originate. These cells have a finite growth capacity and can be subcultured for only a limited number of passages. Subcultured cells, which have been selected to form a population of cells of a single type, are designated cell lines, and can be finite or continuous. Finite cell lines (cells capable of a limited number of generations before proliferation ceases) as well as continuous cell lines can be propagated and expanded for the production of well characterized cell banks, where they are preserved by employing cryopreservation techniques (Doyle *et al.*, 1994).

A normal tissue usually provides finite cultures, while cultures obtained from tumors can result in continuous cell lines (immortal). Nevertheless, there are many examples of continuous cell lines that are obtained from normal tissues and are not tumorigenic, such as BHK 21 (baby hamster kidney fibroblasts), MDCK (Madin-Darby canine kidney epithelial cells), and 3T3 fibroblasts (Freshney, 1994, 2000). Immortal cell lines can occur spontaneously (rarely) or after a transformation process (more often), which can be induced by carcinogenic chemical agents, by viral infection, or by the introduction in the cell genome of a viral gene or an oncogene capable of overcoming senescence. Several of the differences between normal and neoplastic or tumor cells are analogous to the differences between finite and continuous cell lines, since immortalization is an important component of the cell transformation process.

The main advantages of continuous cell lines are: (i) faster cell growth, achieving high cell densities in culture, particularly in bioreactors; (ii) the possible use of defined culture media available in the market, mainly serum-free and protein-free media; and (iii) the potential to be cultured in suspension, in large-scale bioreactors.

The major disadvantages of these cultures are the accentuated chromosomal instability, the larger phenotype variation in relation to the donor tissue, and the disappearance of specific and characteristic tissue markers (Freshney, 1994).

Many examples of immortalization methodologies and techniques to obtain continuous cell lines are described in the literature (Land *et al.*, 1983;

MacDonald, 1990; Ruley, 1983), including those involving transfection or infection with viral genes (for instance, the E6 and E7 genes of human papilloma virus, and the SV40T simian virus 40 large T-antigen gene) or virus (such as Epstein–Barr virus and retroviruses). Another strategy is to create hybrid cells resulting from the fusion of a cell with a limited lifespan with a continuous cell. This is the strategy used to obtain hydridomas for antibody production, as discussed below (see also Chapter 17).

The hybridomas were, to a large extent, responsible for the biotechnology ''explosion'' towards the end of the 1970s, opening perspectives for remarkable advances in both the immunotherapy and diagnostic areas. In 1975, Köhler and Milstein demonstrated that, despite the impossibility of cultivating differentiated B lymphocytes *in vitro*, it was possible after their fusion with immortal myeloma cells. The hybrid cell lines (hybridomas) can grow continuously and produce and secrete immunoglobulins. Since all the immunoglobulin produced derives from a single type of cell, the antibody is monoclonal and is directed against only one epitope. Although the initial studies performed by Köhler and Milstein were restricted to the production of mouse mAbs, soon thereafter the production of antibodies from other species, including human, became possible.

As mentioned before, another relevant key development for the progress of animal cell culture technology was the WI-38 human diploid cell line obtained by Hayflick and Moorhead in 1961, since previously the options were the use of primary cultures or of heteroploid cell lines (derived from tumors or from cells that acquired tumor-like characteristics in culture). Because of that, heteroploid cells were not acceptable for the production of compounds for human applications, and therefore primary cultures from other species were employed (such as primary monkey kidney cells).

After the development of diploid cell lines, i.e. with a diploid karyotype, a new concept of cell line emerged, since these cells are considered 'normal' cells. They undergo senescence and die in culture after a finite number of generations (around 50 generations for WI-38 cells). The disadvantages of these cells in culture are that they grow slowly, not reaching high cell densities, present relatively low productivity, are highly dependent on support adhesion for growth, and consequently are not easily cultured in suspension. Nowadays, the diploid cell line most frequently employed is MRC-5, and its use is particularly important for the study of cell aging mechanisms.

The animal cell lines mentioned above are more extensively discussed in Chapters 2, 17, 18, 20, and 21.

1.3 Use of animal cells in commercial production

1.3.1. Animal cell proteins in human diagnosis and therapy

mAbs are currently the most important class of pharmaceutical proteins in terms of market volume. Given their enormous biological specificity it is not surprising that their first clinical applications were the so-called immunoassays, such as the ELISA-type assays, for *in vitro* diagnosis. After 1980, however, mAbs also started to be used in association with radio-

active markers, in imaging methods, such as immunoscintillography. In addition, with higher doses of the radioactive agent used for tumor detection, it became possible to treat cancer. This is the 'magic bullet' concept initially proposed by Paul Ehrlich at the end of the 19th century. In this application, the antibody directs the radioactive product only to cancer cells, which express large amounts of surface tumor antigens. Instead of radioactive compounds, lymphokines or toxins can also be associated to the antibodies to cause the death of tumor cells.

The first therapeutic antibody approved (Orthoclone® OKT-3® or Muromonab CD3, 1986) was indicated not for cancer treatment, but for controlling acute rejection of transplanted organs (kidney, heart, and liver). Nowadays, other clinical indications such as asthma, rheumatoid arthritis, psoriasis, and Crohn's disease are treated with mAbs (see Chapter 17) (Antibody Engineering and Manufacture, 2005; Monoclonal Antibodies and Therapies, 2004; Hot Drugs, 2004; Walsh, 2004).

Many recombinant proteins that are not antibodies are also on the market for distinct applications (see Chapter 16). Examples are factor VIII for hemophilia A treatment (Bayer, 1993, produced from BHK cells), erythropoietin as an anti-anemic agent (Amgen, 1989, produced from CHO cells) and β-interferon for the treatment of multiple sclerosis (Biogen and Serono, 1996, produced from CHO cells).

Nevertheless, in the last 20 years, the relevance of clinical diagnosis has increased significantly, forming the basis for the pharmaceutical strategy known as pharmacogenomics, which could in the future enable a complete customization/individualization of pharmaceuticals. A direct connection between diagnosis and treatment could make it possible to introduce individualized products that were eliminated for general use due to side effects that were severe, but occurred only in a limited number of patients. This would be acceptable provided that unequivocal diagnostics can be developed to identify these patients. This process has already been adopted sometime ago, for instance, to guarantee that patients who are allergic to an antibiotic, such as penicillin, are not treated with it. However, individualized medical treatment may become more and more frequent to insure that expensive biopharmaceuticals are administered only to patients who can benefit from them and avoiding excessive health expenses. One example is the biopharmaceutical 'trastuzumab' (Herceptin®, Genentech), used for the treatment of a very aggressive breast tumor type that overexpresses the epidermal growth factor receptor type 2 (HER2). This situation occurs in only about 25% of breast cancer cases, so only these patients are treated with trastuzumab.

The value of therapeutic mAbs marketed in 2004 was above US$ 13 billion. At the same time, the market value for the many presentation forms of erythropoietin exceeded US$ 8 billion, and the global biopharmaceutical market surpassed US$ 50 billion.

It is worth emphasizing that all biopharmaceuticals mentioned here are produced from mammalian cell culture. The protein production system based on insect cells known as BEVS (baculovirus expression vector system) is widely employed for the expression of a wide range of proteins, but, due to regulatory issues, biopharmaceuticals produced by insect cells are not yet in the market. However, some of them are being evaluated,

particularly where supramolecular structures designated VLPs are produced (see Chapter 18).

1.3.2 Cell therapy

In the last two decades, the knowledge about stem cells and, specifically, their expansion and differentiation capabilities has grown significantly. These properties (expansion and differentiation) make stem cells unique tools for the treatment of a wide range of diseases, for which traditional therapies have failed or do not exist. Some of the current applications of stem cells are listed in *Table 1.2*.

However, to use these cells in clinical protocols, it is necessary to understand the nature and properties of stem cells originating from different tissues, as well as the mechanisms that make them differentiate into mature, functional cells (Mayhall *et al.*, 2004).

The use of hematopoietic stem cells in bone marrow transplants has paved the way for other therapies, including transplants of skin, pancreatic, and brain cells (Bonner-Weir and Weir, 2005; Laflamme and Murry, 2005). The fact that stem cells have been identified both in systems with high (e.g. blood and skin) and reduced (e.g. brain) regeneration capacity suggests that specific stem cells could also be found in many other organs and tissues. The isolation, expansion, and differentiation of these cells, which could in the future make the treatment of degenerative monocellular disorders (e.g. Parkinson's disease) possible, provides a great challenge for the field of animal cell culture.

As an alternative to adult stem cells, embryonic stem cells can be used. These are totipotent and can be obtained from the internal blastocyst cell mass. Because of the capacity of these cells to generate any type of functional cell, their manipulation and differentiation have gained in significance. In spite of recent advances (Daley, 2003; Hwang *et al.*, 2004), knowledge on the control of their differentiation and proliferation is still lacking, but will be necessary to make the exploitation of all their therapeutic potential turn into reality. Further discussion on cell therapy can be found in Chapter 20.

Table 1.2 Examples of current applications of stem cells

Source	Cell type	Applications
Bone marrow	Hematopoietic	Cancer Immunodeficiencies Metabolic diseases Hemoglobinopathies Myocardial infarction
Neuronal embryonic tissue	Neuronal	Parkinson's disease
Skin	Epithelial	Burns Ulcers Genetic skin diseases
Pancreas	Pancreatic	Diabetes

1.3.3 Tissue engineering

Tissue engineering is a recent discipline that is closely related to cell therapy, but combines knowledge of molecular and cell biology with traditional concepts from biomaterials engineering, bioreactors, biomechanics, and controlled drug release, aiming at the development of new tissues.

The success of tissue engineering depends on the combination of different factors (Heath, 2000). The first one is to have cells that are capable of regenerating and, if necessary, an adequate supporting matrix. Recent developments indicate that it may be possible that the cells themselves manage to build the tissue structure, without the need for an external matrix. Another requirement is an environment that is appropriate for cell growth, differentiation, and eventual integration into the surrounding tissue. Since cells are a critical factor, the source from which they originate deserves special attention. Obtaining a sufficient amount of the appropriate cells is of extreme relevance for the regeneration of damaged tissues, particularly in cases when the tissue does not have the ability to self-regenerate or when the native regeneration mechanisms are not sufficient.

There are different cell sources for use in tissue engineering, each of them presenting advantages and disadvantages. The best source, in principle, is the patient. The autologous cells so obtained are expanded *in vitro* for a later transplant, with no risk of immune reaction. However, the amount of cells obtained from the patient is frequently very limited, especially in cases when cell collection may cause death or in the case of elderly patients. An alternative is the use of stem cells, particularly those that already present some degree of differentiation, but still retain some pluripotency. Although rare, such cells can be propagated and can be used later for reconstituting and repairing the damaged tissue.

The first tissue engineering product to be approved targeted the treatment of burns and consisted of keratinocytes cultivated *in vitro*, which form a tissue that is later transplanted to the patient. Beyond this product, others are already being commercialized for a wide range of applications. Examples are Carticel® (marketed by Genzyme), which consists of chondrocytes employed in the treatment of cartilaginous defects caused by acute or repetitive traumas; Apligraf® (Novartis), used in the treatment of venous ulcer; and DACS® SC (Dendreon), utilized for reconstitution of the immune system after chemotherapy.

In terms of the *in vitro* cell culture techniques required to obtain tissues, the greatest challenges are somehow similar to those of cell therapy, particularly when cells that are not completely differentiated are used. The challenges include the isolation of progenitor cells from the tissue of interest, the expansion of pure cultures, and the understanding of the signals necessary to direct differentiation of a specific cell type, in order to accelerate the process of repairing the tissue. Also in tissue engineering the use of embryonic stem cells is possible, but the control of differentiation is equally an area where a deeper knowledge is still required. It is necessary to assure that the cells introduced into the patient are completely differentiated, since even a small percentage of undifferentiated cells in a tissue can evolve to produce teratomas.

1.3.4 Gene therapy and DNA vaccines

The introduction and expression of recombinant genes in somatic cells, or gene therapy, is also an important application of animal cell culture. The cells employed in this case may be used at two different levels: in the production of vectors that will be later utilized *ex vivo* or *in vivo* or as already transformed cells, which are used directly *in vivo* in the therapy of diseases.

The transfer of genes has been demonstrated both for viral vectors (such as retrovirus, adenovirus, and lentivirus) and nonviral vectors (plasmid DNA and liposomes). Animal cells are used mainly in the production of viral vectors, which present a much larger integration efficacy than non-viral vectors. In this case, the situation is quite similar to the production of proteins. The cells can produce a virus constitutively (retrovirus and lentivirus) (Merten *et al.*, 2001) or serve as a host for their production (adenovirus and adeno-associated virus) (Ferreira *et al.*, 2005). The production of viral vectors for gene therapy usually requires significant genetic modifications of the cells, since these vectors must not be self-replicating, so as not to infect the patient upon therapy. Thus, the producing cells have the genetic information coding for all elements necessary for the construction of the virus, but the vector just contains the information necessary for the transfer of the therapeutic gene to the target cell. The vectors produced can later be used *in vivo*, for instance in the treatment of tumors (Shah *et al.*, 2003) or *ex vivo* for the transformation of cells that will later be introduced into the patient (Aiuti *et al.*, 2003).

From a prophylactic perspective, the same viral vectors can be utilized to transfer a gene coding for an immunogenic protein of a given pathogenic agent (viral or bacterial). This is the case of the so-called DNA vaccines, where an immunogenic protein begins to be produced by the host organism and, thus, a situation similar to the case of an infection occurs (Whalen, 1996). This type of vaccine is discussed more extensively in Chapter 18.

For gene therapy, hematopoietic stem cells are the most common targets due to their capacity for expansion and differentiation. The current applications focus essentially on diseases caused by a localized genetic deficiency. Excellent candidates for gene therapy are those targeting severe combined immunodeficiency (SCID) (Cavazzana-Calvo *et al.*, 2000) and adenosine deaminase deficiency (ADA) (Aiuti *et al.*, 2003), since both affect blood cells and are usually treated through bone marrow transplants (Noguchi *et al.*, 1993). In these cases, the progenitor cells transformed with the respective functional genes complement the immune system of patients. See Chapter 21 for a more extensive discussion on gene therapy.

1.3.5 Applications of animal cells in the development of new products

The majority of the products currently in use are formed by chemical agents that can be potentially dangerous for human health. About 80% of cancer cases are attributed to chemical products found in the environment

(industrial chemicals, cosmetics, food additives, drugs, dyes, etc.). Every year thousands of new products are developed, which need to be adequately tested for toxicity. In February 2001, the European Commission adopted a 'White Book' defining a European strategy for chemical products. One of the aims refers to tests that do not use animals.

Nowadays, the field of toxicology uses different *in vivo* models (mouse, rat, rabbit, among others) to evaluate the toxic potential of chemical compounds. These classical tests using animals present significant limitations regarding the applicability of the results to the case of humans, and pose ethical problems due to the use of animals. The application of the current European law implies the sacrifice of 12 million animals, corresponding to € 20 billion by 2012.

The development of tests that use cells cultured *in vitro* instead of animals could thus represent an important alternative in this area. Furthermore, the use of cell lines and primary tumor cells allows the evaluation of the potential of anti-tumor compounds with no need for inducing tumors in animals. The current protocols are well established for a variety of tumors, for instance in the National Cancer Institute of the USA.

Confidence in *in vitro* tests represents one of the greatest challenges. In this type of test, the use of human cells in culture is especially promising for the evaluation of acute and chronic toxicity. Current tests are based on the growth-inhibiting potential of the chemical compound, as briefly presented in Chapter 2. The current methods include the determination of proteins or ATP, or the incorporation of an indicator (neutral red), and these analyses can be combined with an evaluation of cell morphology. More recently, efforts have been made to use specific cells and incorporate gene expression analysis in order to have a more precise determination of sublethal toxicity to specific organs. At the same time, the development of high-throughput systems has contributed to even greater possibilities for this type of assay.

The sequencing of the human genome has allowed an increase in the number of therapeutic targets from around 500 to about 1000, paving the way for a qualitative improvement not only in the development of new candidate products, but also in the efficacy of the therapeutic product. Recent advances in areas such as genomics and proteomics allow the association of the genetic information to new proteins. Thus, the understanding of disease mechanisms and their relation to the genes involved could be used to predict the possibility of acquiring diseases and also to offer possible therapies. In this respect, it is expected that new technologies and strategies involving animal cells will be developed, allowing the determination of the security and efficacy of new products with greater accuracy. These will include tests for the bioavailability of products, their eventual genomic impact and the regulation of target genes, among others, with applications to research and the development of new pharmaceuticals, nutraceuticals, or cosmetic products.

1.4 Conclusions

A significant number of industrial cell lines (CHO, BHK, NS2/0) are nowadays well characterized, forming the basis for mature technologies

for the production of recombinant proteins, for both therapeutic and diagnostic purposes.

Other uses for obtaining more complex products, such as viruses for vaccines, are also well established. However, the use of animal cell technology for applications such as viral vectors for gene therapy, anti-cancer vaccines, cell therapy, or regenerative medicine (organ and tissue engineering), is in a much more initial phase, since more scientific work is needed to provide improved technologies and respond to complex regulatory and ethical questions.

Finally, the most recent 'stars' of this area, the stem cells, are still in their infancy, due to the greater biological complexity that they represent. Their potential uses are so promising, however, that this will assure a great and continuous effort to determine the controlled conditions needed for expansion, complete differentiation, and storage, probably in hospital environments.

If we add to all these applications and challenges the dominant position of animal cell technology in the discovery and development phases of new pharmaceuticals, it is easy to predict two or three decades of continued expansion in this field of knowledge and technology.

References

Adams GP, Weiner LM (2005), Monoclonal antibody therapy of cancer, Nat. Biotech nol. 23:1147 1157.

Aiuti A, Ficara F, Cattaneo F, Bordignon C, Roncarolo MG (2003), Gene therapy for adenosine deaminase deficiency, Curr. Opin. Allergy Clin. Immunol. 3:461 466.

Alves PM, Moreira JL, Rodrigues JM, Aunins JG, Carrondo MJT (1996), Two dimensional versus three dimensional cultures systems: effects on growth and productivity of BHK cells, Biotechnol. Bioeng. 52:429 432.

Antibody Engineering and Manufacture (2005), Special issue Nat. Biotechnol., vol. 23.

Bonner Weir S, Weir GC (2005), New sources of pancreatic beta cells, Nat. Biotech nol. 23:857 861.

Cavazzana Calvo M, Hacein Bey S, Basile GS, Gross F, Yvon E, Nusbaum P, Selz F, Hue C, Certain S, Casanova J L, Bousso P, Le Deist F, Fisher A (2000), Gene therapy of human severe combined immunodeficiency (SCID) X1 disease, Science 288:669 672.

Cruz PE, Maranga L, Carrondo MJT (2002), Integrated process optimisation: lessons from retrovirus and virus like production, J. Biotechnol. 99:199 214.

Daley GQ (2003), From embryos to embryoid bodies: generating blood from embryo nic stem cells, Ann. Acad. Sci. 996:122 131.

Doyle A, Griffiths JB, Newel DG (1994), Cell & Tissue Culture: Laboratory Procedures, John Wiley and Sons, New York, pp. 3:01 3:03.

Ferreira TB, Alves PM, Aunins JG, Carrondo MJT (2005), Use of adenoviral vectors as veterinary vaccines, Gene Ther. 12: S73 S83.

Finter NB, Chapman S, Dowd P, Johnston JM, Manna V, Sarantis N, Sheron N, Scott G, Phua S, Tatum PB (1991), The use of interferon alpha in virus infections, Drugs 42:749 765.

Freshney RI (1994), Culture of Animal Cells. A Manual of Basic Techniques, 3rd Ed., John Wiley and Sons, New York, pp. 9 16.

Freshney RI (2000), Introduction to basic principles, In: Masters JRW (Ed.), Animal Cell Culture: A Practical Approach, Oxford University Press, New York, pp. 1 16.

Griffiths JB (1988), Cell products: an overview, In: Spier RE, Griffiths JB (Eds), Animal Cell Biotechnology, Vol. 3, Academic Press, London, pp. 179 221.
Hayflick L, Moorhead PS (1961), The serial cultivation of human diploid cell strains, Exp. Cell Res. 25:585 621.
Heath CA (2000), Cells for tissue engineering, Trends Biotechnol. 18:17 19.
Hot Drugs (2004), Nat. Rev. Drug Discov., Suppl. Vol. 3.
Hwang WS, Ryu YJ, Park JH, Park ES, Lee E, Koo, JM, Jeon HY, Lee BC, Kang SK, Kim SJ, Ahn C, Hwang JH, Park KY, Cibelli JB, Moon SY (2004), Evidence of a pluripotent human embryonic stem cell line derived from a cloned blastocyst, Science 303:1669 1674.
Kohler G, Milstein C (1975), Continuous cultures of fused cells secreting antibody of predefined specificity, Nature 256:495 497.
Laflamme MA, Murry CE (2005), Regenerating the heart, Nat. Biotechnol. 23: 845 856.
Land H, Parada LF, Weinberg RA (1983), Tumorigenic conversion of primary fibroblasts requires at least two co operating oncogenes, Nature 304:596 602.
MacDonald C (1990), Development of new cell lines for animal cell biotechnology, CRC Crit. Rev. Biotechnol. 10:155 178.
Mayhall EA, Paffett Lugassy N, Zon LI (2004), The clinical potential of stem cells, Curr. Opin. Cell Biol. 16:713 720.
Merten O W, Cruz PE, Rochette C, Geny Fiamma C, Bouquet C, Gonçalves D, Danos O, Carrondo MJT (2001), Comparison of different bioreactor systems for the production of high titer retroviral vectors, Biotechnol. Prog. 17:326 335.
Monoclonal antibodies and therapies (2004), Supplement to Nature Publishing Group, http://www.nature.com/focus/antibodies/index.html, accessed in February 2006.
Noguchi M, Yi H, Rosenblatt HM, Filipovich AH, Adelstein S, Modi WS, McBridge OW, Leonard WJ (1993), Interleukin 2 receptor gamma chain mutation results in X linked severe combined immunodeficiency in humans, Cell 73:147 157.
Powers MJ, Janigian DM, Wack KE, Baker CS, Beer Stolz D, Griffith LG (2002), Functional behavior of primary rat liver cells in a three dimensional perfused microarray bioreactor, Tissue Eng. 8:499 513.
Ruley HE (1983), Adenovirus early region 1A enables viral and cellular genes to transform primary cell in culture, Nature 304:602 606.
Shah AC, Benos D, Gillespie GY, Markert JM (2003), Oncolytic viruses: clinical applications as vectors for the treatment of malignant gliomas, J. Neurooncol. 65:203 226.
Spier RE (2000), History of animal cell technology, In: Spier RE (Ed.), Encyclopedia of Cell Technology, John Wiley & Sons, New York, v. 2, pp. 853 872.
Walsh G (2004), Modern antibody based therapeutics, BioPharm. International, December, 18 25.
Whalen, R.G. 1996. DNA vaccines for emerging infectious diseases: What if? Emerg. Infect. Dis. 2: 168 175.
Witkowski JA (1979), Alexis Carrel and the mysticism of tissue culture. Med. Hist. 23:279 296.

Animal cells: basic concepts

2

Patrícia Léo, Adriana Lages Lima Galesi, Cláudio Alberto Torres Suazo, and Ângela Maria Moraes

2.1 Introduction

The term cell culture refers to the cultivation of dispersed cells taken from an original tissue, a primary culture, or a cell line. As mentioned in Chapter 1, the practice of cultivating cells started at the beginning of the 20th century and was developed from a simple exploratory phase to an expansion phase in the 1950s. For more than 50 years, the culture of cells derived from primary tissue explants has predominated, justifying the original name "tissue culture." Currently, cell culture is in a specialization phase. With the increase of the use of dispersed cells since the 1950s, the term tissue culture was substituted by cell culture.

At the present time, cell culture techniques allow *in vitro* propagation of various cell lines including those from insects, humans, mice, rats, and other mammals. Basically, animal cell culture techniques are similar to those employed for bacteria, fungi, and yeast, although there are some characteristic differences. In general, animal cells are more delicate, vulnerable to mechanical damage, present lower growth rates, and require more complex culture media and special substrates (Augusto and Oliveira, 2001). Moreover, cell culture has to be performed under rigorous aseptic conditions, since animal cells grow more slowly than most usual contaminants, such as bacteria and fungi.

The goal of this chapter is to discuss animal cell culture characteristics, focusing on typical cell structural and morphological aspects, the establishment, maintenance, and storage of cell lines, *in vitro* cell growth phases, the effects of environmental conditions on cell cultivation, and culture of anchorage-dependent cells, with emphasis on maintaining cell lines employed for the production of commercially attractive biomolecules. Cell cycle phases (G0, G1, S, G2, and M), through which cells pass during exponential growth, are discussed in detail in Chapter 7.

2.2 Typical structure of an animal cell

Various animal cell lines can be cultivated *in vitro*, such as cardiac cells, fibroblasts, smooth muscle cells, endocrine cells (such as pituitary, adrenal, and pancreatic cells), epithelial cells (such as liver, mammary, lung, and kidney cells), tumor cells (such as melanocytes), nervous system cells (such

as glial cells and neurons), as well as hybridomas. Although these cells have different origins and functions, they all show a typical structure represented in *Figure 2.1*.

Animal cells have two very distinct compartments, the cytoplasm and the nucleus, with a constant flow of distinct chemical compounds between them. While the cytoplasm is enclosed in a plasma membrane, the nucleus is enclosed by a nuclear envelope. The different organelles of the cell are immersed in the cytoplasm. Each of these cellular components has specific characteristics and functions that will be discussed below.

2.2.1 Plasma membrane

The cell membrane, also called the plasma membrane, surrounds the cytoplasm and physically separates the intracellular components from the extracellular environment. It has a thickness around 7.5–10 nm and controls the flux of molecules between the cytosol and the extracellular environment. The plasma membrane is composed of a lipid bilayer, in which the polar groups are exposed to the internal and external cell aqueous medium, protecting the embedded hydrophobic tails. Various proteins with distinct functions can be found partially or totally immersed in this bilayer.

The lipid bilayer has two important properties. First, its interior hydrophobic environment acts as an impermeable barrier that prevents the

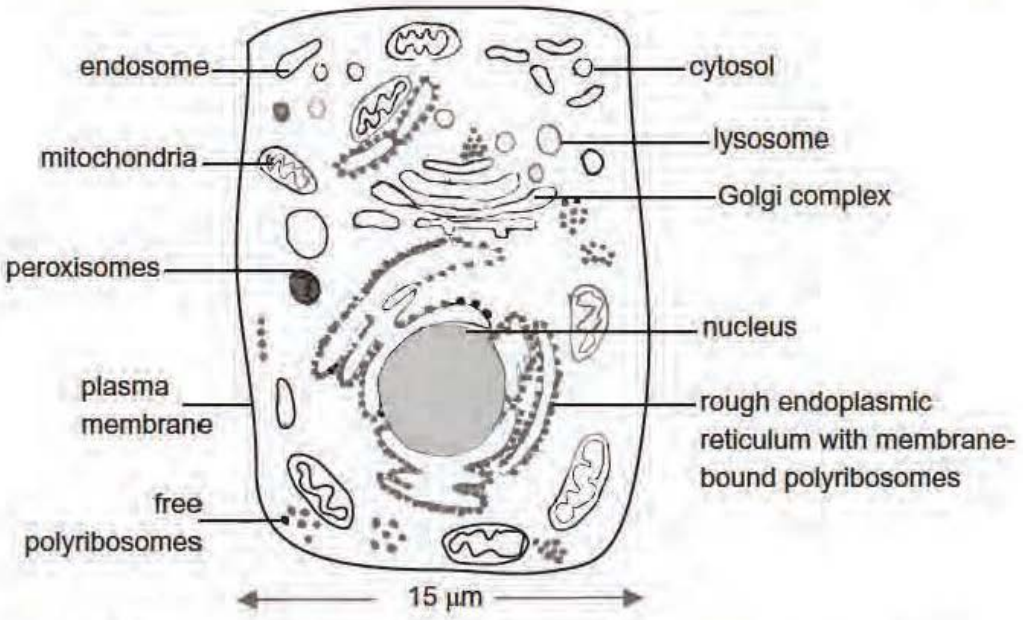


Figure 2.1

Main intracellular compartments of an animal cell. Cytosol, endoplasmic reticulum, Golgi complex, nucleus, mitochondria, endosome, lysosome, and peroxisome are distinct compartments that are isolated from the rest of the cell by at least one selective membrane.

diffusion of hydrophilic solutes through the membrane. This impermeability is selectively modulated by the presence of membrane proteins, which mediate the transport of specific molecules through the plasma membrane. The second bilayer property is its structural stability, maintained by hydrophobic and van der Waals interactions formed between the lipid chains. Even if the external aqueous environment varies with respect to ionic strength and pH, the lipid bilayer has the capacity to maintain its characteristic architecture.

2.2.2 Cytoplasm

The eukaryotic cell cytoplasm consists of the cytosol, which is the matrix that presents no visible structure even when observed by electron microscopy. Organelles and inclusion bodies are immersed in the cytosol. The organelles are responsible for intense metabolic activity and include the mitochondria, Golgi complex, lysosome, and endoplasmic reticulum (*Figure 2.1*). Inclusion bodies are less frequent and consist of lipid, carbohydrate, and pigment-storing structures.

The cytoplasm usually has a thin peripheral area in contact with cell membrane, termed the ectoplasm. This area has few organelles and inclusion bodies, which tend to be located in the central part of the cytoplasm, named the endoplasm.

Although the basic architecture of all eukaryotic cells is formed by membranes, organelles, and cytosol, each cell type exhibits a distinct morphology defined by cell shape and localization of organelles. The structural basis of the characteristic morphology of each cell type is the cytoskeleton, a dense network of three classes of filamentous proteins that permeate the cytosol and support the cell membrane.

2.2.3 Endoplasmic reticulum

This is an interconnected network of flattened or spherical vesicles and tubules found in the cytoplasm of eukaryotic cells. These structures are enveloped by a membrane that separates the endoplasmic reticulum cavities or cisternae. The cisternae constitute a network of channels that go through the cytoplasm and regulate the transport of various cell products, generally to the exterior environment. In some cells the cisternae also serve as a storage area. There are two types of endoplasmic reticulum: granular (or rough) and smooth.

The rough endoplasmic reticulum contains ribosomes on the surfaces, a cell structure that will be discussed in the next section. The system formed by endoplasmic reticulum and ribosomes is associated with protein synthesis. The endoplasmic reticulum also participates in lipid biosynthesis. In different types of eukaryotic cells, the endoplasmic reticulum has different forms and functions. In muscle cells, in which Ca^{2+} stimulates contraction, the endoplasmic reticulum participates in the relaxation process, reabsorbing Ca^{2+} ions.

The smooth endoplasmic reticulum also consists of a network of tubules that is considerably developed in certain types of special cells, such as those that secrete steroid hormones.

2.2.4 Ribosome

Ribosomes are spherical cell structures enriched with ribonucleoprotein and with a diameter of 15–20 nm. Each ribosome is formed by two subunits of different sizes, only visible through high resolution electron microscopy. Ribosomes connected to the rough endoplasmic reticulum are involved in the biosynthesis of proteins that are temporarily stored or transported to the cell's outer environment. Proteins synthesized by ribosomes connected to membranes accumulate in the cisternae before passage to the plasma membrane for secretion.

2.2.5 Golgi complex

This organelle is also known as the Golgi or Golgi apparatus and is named after the Italian cytologist Camilo Golgi, who was one of the first to study it.

The Golgi has distinct forms in different cell types. The most characteristic arrangement is a stack of circular flattened vesicles, with variable sizes, each one held by a single membrane in which can be found smaller spherical vesicles that bud off from the larger ones. In many cells, the Golgi complex is situated near the cell nucleus but in other cells, it is dispersed in the cytoplasm.

The Golgi complex receives products from the endoplasmic reticulum and packs them in secretory vesicles, which are conducted and fused to the external cell membrane. In this process, termed exocytosis, the vesicle content is secreted into the outer environment.

2.2.6 Mitochondria

Mitochondria are spherical or, more frequently, elongated bodies, consisting of two membrane units. The inner one is folded in the form of tubules or shelves, named cristae.

Mitochondria are enzyme-rich structures that catalyze the oxidation of organic nutrients by oxygen molecules, producing carbon dioxide and water. Some of these enzymes are located in the matrix and some in the internal membranes. Chemical energy is released during these oxidation processes and is used to generate adenosine triphosphate (ATP), the most important cell energy carrier molecule. ATP synthesized by mitochondria diffuses throughout the cell and is utilized for many cellular functions.

The mitochondria also contain small quantities of DNA, as well as RNA and ribosomes. Mitochondrial DNA encodes the synthesis of certain specific inner cell membrane proteins. Mitochondria can also divide during cell replication.

2.2.7 Lysosome

Lysosomes are spherical organelles, enveloped by a membrane and containing several hydrolytic enzymes that present maximal activity at acidic pH. These enzymes are synthesized in the rough endoplasmic reticulum. Lysosomes are reservoirs of different enzymes capable of degrading

macromolecules by hydrolysis such as proteins, polysaccharides, and lipids. As these enzymes may be harmful to other cell components, they remain segregated in the lysosomes. Proteins and other components to be degraded are selectively transported to the lysosomes and are then hydrolyzed to their fundamental units, which are afterwards secreted into the cytoplasm.

2.2.8 Peroxisome

Peroxisomes or microbodies are spherical organelles that are 0.3–1.5 μm in diameter. Each peroxisome is enveloped by a single external membrane, and its interior is full of proteins, frequently in crystalline form. Peroxisomes are characterized by the presence of various oxidative enzymes, which have variable functions dependent upon the origin of the peroxisome. These enzymes generate and utilize hydrogen peroxide (H_2O_2), hence the name peroxisome. This compound is very toxic for cells and is decomposed by the enzyme catalase to water and oxygen.

2.2.9 Nucleus

The nucleus of eukaryotic cells is a very complex structure, containing various components. It is separated from the rest of the cell by two membranes named the nuclear envelope. At regular intervals, the two membranes of the nuclear envelope form pores with a diameter of around 90 nm. These pores regulate flux of macromolecules to and from the cytoplasm. Inside the nucleus is located the nucleolus, which acts to produce ribonucleic acid (RNA), which is the first step for ribosome synthesis.

Chromatin occupies the remaining part of the nucleus and consists of DNA, RNA, and specialized proteins. Between cell divisions, chromatin is dispersed in the nucleus but, immediately before cell division, chromatin is arranged in granular bodies named chromosomes. After replication, daughter chromosomes are separated and distributed to daughter cells during the mitosis process, after which the chromatin is dispersed again.

2.3 Cell culture

2.3.1 Establishing a cell line

Cells can be cultivated *in vitro* after a tissue is dissected and totally or partially disaggregated by enzymic treatment. Cell, explants, or organ cultures can be established depending on the degree of disaggregation of the tissue, as shown in *Figure 2.2*.

Primary cultures are those obtained directly from organ or tissue fragments, following enzymic or mechanical disaggregation. For enzymic treatment, trypsin or collagenase may be used and may be mixed with EDTA (ethylenediaminetetraacetic acid). Primary cultures can originate from animal or human tissue. A culture is considered a primary culture up to its first subculture or passage; after that it is named a cell line. *Figure 2.3*

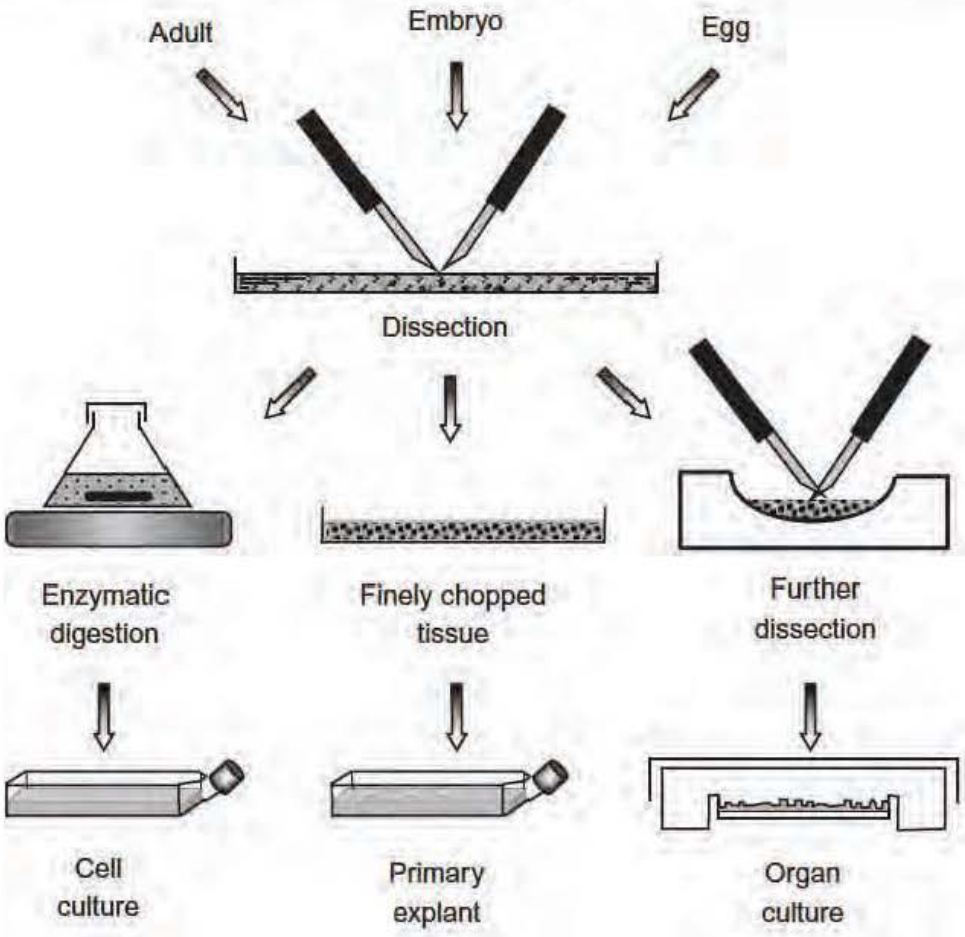


Figure 2.2

In vitro culture types of animal cells (adapted from Freshney, 2000, 2005).

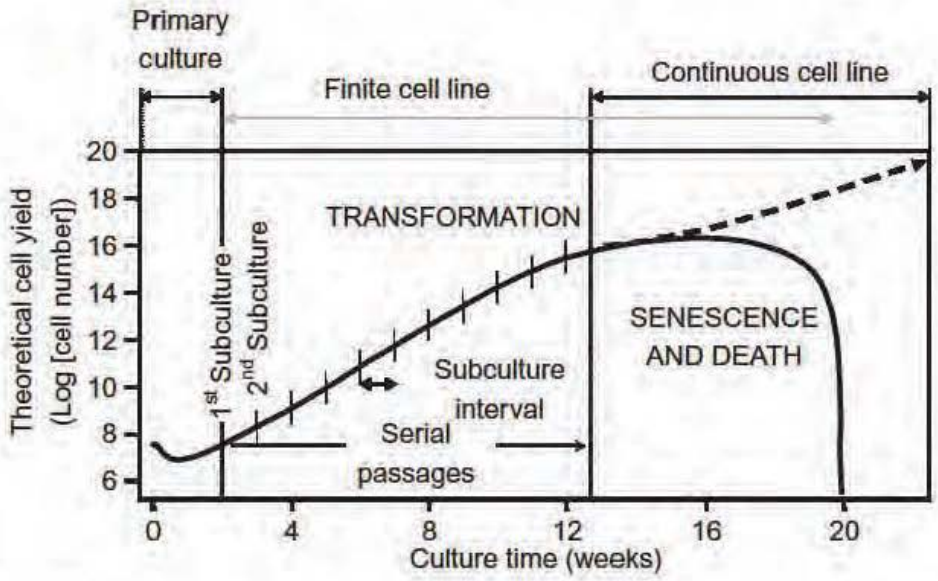


Figure 2.3

Typical evolution of a cell line (adapted from Freshney, 2000, 2005).

shows a schematic diagram of the sequence of events associated with subculture of a normal primary cell culture.

Primary cultures are initially heterogeneous, but fibroblasts may become predominant after some growth. Obtaining primary cultures is laborious and cells can be maintained *in vitro* for only a limited period. During this period, cells generally maintain the differentiated characteristics of the original tissue from which they were harvested.

During primary culture, cells may be subjected to considerable stress. For instance, the enzymatic dissociation of organ fragments or adherent cells breaks cell–cell or cell–surface interactions. Dissociated cells generally change their shape, becoming rounded and losing their phenotypic polarity, modifying protein distribution in cell membrane.

Certainly not all cells survive cell manipulation but those that survive should be able to correct any injuries and adapt to environmental changes. Culture adaptation is time-consuming and is influenced by culture conditions. Cells condition their environment through the release of substances into culture medium, such as growth factors, which promote cell adherence and proliferation. Some cell lines can adapt, proliferate, and differentiate faster if culture medium is previously conditioned by cells in active growth. Environmental conditioning can also be achieved by adding cells to medium already inoculated with a non-mitotic viable cell population, named feeder cells. These cells can be prepared from 3T3 fibroblastic cells exposed to gamma radiation or treated with mitomicin C (Rheinwald, 1989).

After successive subcultures of a very heterogeneous primary culture, containing many cell types of the original tissue, a more homogeneous cell line with a higher growth rate may arise. A cell line can be serially propagated in culture, usually for only a limited number of cell divisions. Finite cell lines are generally diploid and maintain some degree of differentiation. Nevertheless, these cell lines die after a limited number of generations, the Hayflick limit, which is usually about 30–50 division cycles depending on the origin of the cells (Hayflick and Moorhead, 1961).

The immortalization of a cell line can be accomplished as a spontaneous process, by an oncogene or virus or by chemical treatment. This can lead to a continuous cell line that can be propagated for an undetermined period. If such changes occur with an affect on cell cycle control, the cell line can be designated a transformed cell line.

According to Freshney (2000), the main characteristics of transformed cell lines are: (i) altered cell morphology (smaller, less adherent, or more rounded cells, with a higher nucleus to cytoplasm ratio); (ii) higher growth rate (duplication times decrease from 36–48 hours to 12–36 hours); (iii) less dependency on blood serum or selected growth factors; (iv) increase in cloning efficiency; (v) increase in heteroploidy (chromosomal variation between cells) and in aneuploidy (divergence from the original diploid number); (vi) increase in tumorigenicity. Some of these characteristics are associated with malignant transformations. The main advantage of transformed cells for cell culture is the almost unlimited cell supply. However, a disadvantage is that the cells generally maintain few characteristics of their original tissue.

2.3.2 Cell line maintenance

When a primary culture or a cell line is initiated, a periodic change of culture medium is necessary, followed by subculture if the cells are proliferating. Culture medium changes should be performed even in cultures showing no cell proliferation, since cells can metabolize and deplete nutrients from the medium. Also, some nutrients may degrade spontaneously. Intervals between medium changes and subcultures may vary depending on the cell line, growth rate, and metabolism. An increase in cell density, pH decrease, nutrient depletion in the medium, or alteration in cell morphology indicate the need for culture medium replacement.

Animal cells may be anchorage-dependent. Cells that depend on a solid substratum for growth are named adherent cells. These cells normally proliferate in monolayers and show contact inhibition, with the maximum cell yield generally limited by the available surface of the culture vessel. The yield of cells in suspension is not dependent on a solid substratum.

Table 2.1 shows some adherent and suspension cell lines that are commonly cultured. Cell shape usually reflects their origin. Blood cells (such as those derived from lymphomas) generally grow in suspension, while cells derived from solid tissues (such as kidney and liver) are adherent cells. Typical morphologies of different cell types are shown in *Figure 2.4* (see color section).

Since adherent cells proliferate only after cell surface adhesion, an understanding of the steps of this process is very important. The first step consists of the adsorption of adhesion factors to the substratum, such as

Table 2.1 Adherent and suspension cell lines commonly employed in animal cell culture

Name	Cell type and tissue origin	Morphology
Adherent cells		
MRC 5	Human lung	Fibroblast
HeLa	Human cervix	Epithelial
Vero	African green monkey kidney	Epithelial
NIH 3T3	Mouse embryo	Fibroblast
L929	Mouse connective tissue	Fibroblast
CHO	Chinese hamster ovary	Fibroblast
BHK 21	Syrian hamster kidney	Fibroblast
HEK 293	Human kidney	Epithelial
HEPG2	Human liver	Epithelial
BAE 1	Bovine aorta	Endothelial
Suspension cells		
NS0	Mouse myeloma	Lymphoblastoid like
U937	Human hystiocytic lymphoma	Lymphoblastoid
Namalwa	Human lymphoma	Lymphoblastoid
HL60	Human leukemia	Lymphoblastoid like
WEHI 231	Mouse B cell lymphoma	Lymphoblastoid
YAC 1	Mouse lymphoma	Lymphoblastoid
U 266B1	Human myeloma	Lymphoblastoid
SH SY5Y	Human neuroblastoma	Neuroblast

Adapted from ECACC Handbook, 2005.

vitronectin and/or fibronectin glycoproteins and often associated to Ca^{2+} ions. These factors can be derived from serum or can be produced by the cells themselves. The second step consists of contact of the cell with the surface, while in the third step, cells attach to the covered surface, producing multivalent heparin sulfate proteoglycans, which bind to cell membrane glycoproteins. In the fourth step, cell spreading over the solid surface occurs.

As vertebrate cells have negative charges non-uniformly distributed over their external membrane surface, solid substrata with a hydrophilic surface are required, with an adequate distribution of the surface charge.

For the subculture of adherent cells, removal of culture medium and the detachment of cells from the monolayer are necessary. This detachment is usually performed with trypsin, but other proteases, such as pronase, dispase, and collagenase, can be employed. In general, a chelating agent, such as EDTA, is also added to capture the Ca^{2+} ions involved in the cell adhesion process. Some cell lines bind weakly to surfaces and, in small-scale cultures, can be removed mechanically by gently tapping or hitting the culture flask by hand.

For cells growing continuously in suspension, the subculture process can be performed similarly to the method used for microbial cultures. Trypsin treatment is not required and subculture is faster and less traumatic for the cells. Total medium exchange is not generally performed for these cultures since it would require a centrifugation step. Culture maintenance can be performed by dilution with fresh medium after adequate cell growth.

Some cell lines can proliferate as semi-adherent cells, and the population is a combination of suspended and adherent cells. To maintain the heterogeneous nature of such cultures, both suspended and adherent cells should be subcultured.

Suspension cultures present some advantages in comparison with adherent cell cultures. Large cell quantities can be attained without increasing the superficial area of the substratum. Also, a steady state can be reached if nutrients are continuously supplied and cell concentration is kept constant. This can rarely be achieved with a monolayer culture. Cells in different modes (adherent or suspension) behave differently with regard to proliferation, enzymatic activity, glycolysis, respiration, specialized product synthesis, and many other properties.

2.4 Cell growth phases

Normal cells in culture show a sigmoid pattern of proliferative activity that reflects culture adaptation, environmental conditioning, nutrient availability and, for adherent cells, available free adhesion surface.

Cell growth phases comprise lag phase, exponential or log growth phase, stationary or plateau phase, and senescence or death phase, as shown in *Figure 2.5*. Cell growth can be mathematically represented by the following general equation:

$$\frac{dX}{dt} = \mu.X \qquad (1)$$

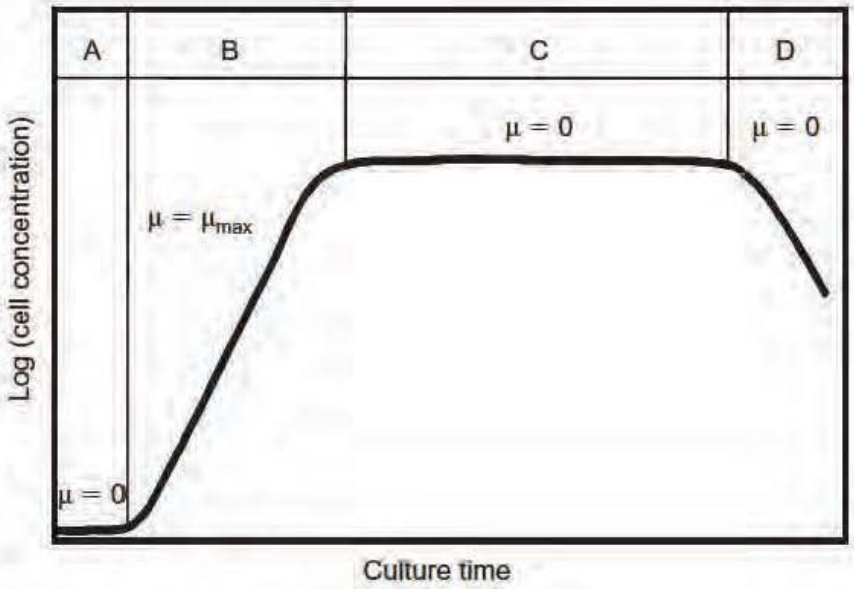


Figure 2.5

Normal animal cell growth curve pattern, in which μ is the specific cell growth rate. Lag phase (A) represents the culture adaptation period, followed by an exponential cell growth phase (B) until the attainment of a stationary or plateau phase (C), in which there is no increase in cell number. The culture reaches the senescence phase (D) when the percentage of cells in division becomes lower than the percentage of cells dying.

where μ refers to specific cell growth rate, X is cell concentration and t is the culture time.

The lag phase occurs after cell inoculation. In this phase, there is no cell division or division takes place at low specific rates. It is an adaptation period in which adherent cells may resynthesize the glycocalyx elements lost during trypsinization, bind, and spread on the substratum. During spreading, the cytoskeleton reappears and new structural proteins are synthesized (Freshney, 2005). The duration of the lag phase is dependent on at least two factors: the point in the growth phase from which cells were taken in the previous culture and the inoculum concentration. Cells originating from an actively growing culture have a shorter lag phase than those from a quiescent culture. Cultures initiated at low cell densities condition the culture medium more slowly and hence increase the duration of the lag phase, which is not desirable.

Exponential or log phase is a period of active proliferation during which the cell number increases exponentially. In the log phase, the percentage of cells in division can reach 90–100%, and cells are in their best physiological state, which is ideal for cell function studies. During the log phase, there is a kinetic profile typical of all cell lines. The cell doubling time (t_d) can be determined through the direct integration of equation 1, since μ is constant and, in this phase, attains a maximum value (μ_{max}), resulting in:

$$t_d = \frac{\ln 2}{\mu_{max}} \tag{2}$$

Factors that influence the duration of the log phase are inoculum concentration, cell growth rate, nutrient availability, and accumulation of inhibitory metabolites. For adherent cells, the end of the log phase may also occur at confluence, when cells cover all the available growth surface, at which point contact inhibition restricts further growth.

During the stationary or plateau phase, cell growth rate is reduced due to low nutrient concentrations and the accumulation of inhibitory metabolites. In some cases, proliferation almost completely ceases. During this phase, cell division is equilibrated with cell death, and the percentage of cells in division is at most 10%. At confluence the growth of adherent cells is inhibited by cell to cell contact but a certain degree of mitotic activity may still be observed. Cells occupy a smaller surface area, exposing less of their own membrane surface to the culture medium. A relative increase in specialized protein synthesis (as opposed to structural proteins), as well as a change in cell surface composition and charge modification may occur (Freshney, 2005). The stationary phase may be prolonged if the culture medium is replenished with fresh medium. This is not a stable period for most cell lines, and they are more susceptible to injuries.

The stationary phase is followed by a decline period in which cell death is not compensated by cells in proliferation. Cell death can occur by two distinct mechanisms, named necrosis and apoptosis. Necrosis occurs as a result of an irreversible injury and normal homeostasis is lost. *In vivo*, this form of death generally affects the neighboring cells and may result in inflammation. Autodestruction occurs by activation of hydrolases when there is a lack of nutrients and oxygen, followed by progressive disorganization and complete disintegration of the cell.

Apoptosis, on the other hand, occurs through the activation of a biochemical program involving a cascade of cell components, which is internally controlled, requiring energy and not involving inflammation *in vivo*. The most frequently observed biochemical events during apoptosis comprise caspase activation, mitochondrial membrane permeation, leakage of diverse molecules from the mitochondria, nuclease activation, cytoskeleton destabilization, externalization of phosphatidylserine to the outer membrane, and protein interconversion. This topic is discussed more extensively in Chapter 7.

The determination of the cell growth profile is important to evaluate the specific characteristics of a cell line culture. Cell behavior and biochemistry are significantly altered in each growth phase. Hence, knowing the growth curve of each cell line is important for establishing the most adequate inoculum concentration, prediction of the length of an experiment, and the most appropriate time intervals for sampling.

Cell concentration in suspension can be determined through an optical microscope employing a hemocytometer for manual cell counting, or in a semi-automatic way using an electronic particle counter (such as a Coulter counter), as described in detail by Freshney (2005). Through dye exclusion (such as trypan blue), it is possible to determine viable cell concentration, that is the number of cells in a known sample volume capable of proliferating in favorable culture conditions.

2.5 Influence of environmental conditions on animal cell culture

Cell culture systems must provide the physiological conditions for cell survival and proliferation. *In vitro*, animal cell growth is dependent on several factors, such as pH, temperature, osmolality, gas concentration (oxygen and CO_2), available surface substrata, and state of the cells at inoculation (Freshney, 2005). Other factors that impact the culture are medium composition, which can differ extensively between cell lines and is discussed in detail in Chapter 5, as well as susceptibility to hydrodynamic stress, as discussed in Chapter 7.

2.5.1 pH

pH control is fundamental for *in vitro* cell culture. Most mammalian cell lines proliferate at pH 7.4. Although the optimal pH value for cell growth does not vary too much for different cell lines, some normal fibroblasts proliferate well at a pH range between 7.4 and 7.7, and transformed cells have an optimal growth at a pH varying from 7.0 to 7.4. Insect cells show better proliferation at lower pH values, from 6.2 to 6.5.

It is possible to monitor pH variation by adding a pH indicator. The compound most used is phenol red, which is rose-colored at pH 7.8, red at pH 7.4, orange at pH 7.0, and yellow at pH 6.5. Nevertheless, it is convenient to point out that most commercially available phenol red contains impurities that could influence cell behavior. Also, this compound can interfere with the interpretation of experimental data obtained by the use of fluorescence and absorbance techniques.

Culture medium needs to be buffered to compensate for CO_2 and lactic acid derived from glucose metabolism. Most culture media employed for animal cells are buffered with CO_2 originating from the gaseous phase, in equilibrium with sodium bicarbonate ($NaHCO_3$) added to the culture medium, as described by the following equilibrium reaction:

$$CO_2 + H_2O \leftrightarrow H_2CO_3 \leftrightarrow H^+ + HCO_3 \quad (3)$$

$$NaHCO_3 \leftrightarrow Na^+ + HCO_3 \quad (4)$$

An increase in CO_2 tends to increase H^+ and HCO_3 in reaction (3), consequently increasing medium acidity. In compensation, the increase in HCO_3 causes $NaHCO_3$ formation through reaction (4), until an equilibrium is reached at pH 7.4. In summary, a medium pH decrease due to a CO_2 increase in the gas phase is neutralized by the action of sodium bicarbonate, with the pH stabilizing at 7.4. Traditionally, culture media are buffered with sodium bicarbonate at a final concentration of 24 mM.

When cells are growing at low densities or are in a lag phase, they do not produce CO_2 in sufficient quantities to maintain the pH at an optimal value. Decreasing the CO_2 content in the gaseous phase results in an increase in the culture equilibrium pH. Therefore, the control of CO_2 concentration allows appropriate maintenance of culture pH (Kilburn, 1991).

This type of buffering is of low cost, non-toxic, and also provides other chemical benefits for the cells. Each basal medium has its own bicarbonate

concentration and CO_2 tension recommended to attain the correct pH and osmolality values. *Table 2.2* indicates the recommended $NaHCO_3$ and CO_2 concentrations for a set of commonly employed basal media. The gas phase in contact with cell culture in an incubator is usually adjusted to 5–10% of CO_2 in volume (90–95% air).

In some situations, the utilization of a system with a higher buffering capacity is needed. In this case, organic buffers can be employed, and in this category, the most widely used is Hepes (N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid). Buffering with Hepes is most effective at the 7.2–7.6 pH range, and this compound is more resistant to rapid alterations in culture conditions. When using Hepes, a controlled gaseous atmosphere for mammalian cell growth is not required. Some media are efficiently buffered by Hepes and bicarbonate mixtures. Nevertheless, Hepes is relatively expensive, and is toxic for cells in concentrations above 100 mM.

For insect cells, culture medium is buffered with sodium phosphate, and the use of CO_2 or pH indicators is not required.

Table 2.2 Recommended values of $NaHCO_3$ in the liquid phase and CO_2 concentration in the gas phase to be employed with traditional basal media

Basal medium	$NaHCO_3$ concentration in medium (mM)	CO_2 percentage in gaseous phase
Mammalian cells culture media		
Eagle MEM*	4	Atmospheric air
IMDM	36	5
TC199	26	5
DMEM/Ham's F12	29	5
RPMI 1640	24	5
Ham's F12	14	5
DMEM	44	10
Insect cells culture media		
Grace*	4	Atmospheric air
IPL 41*	4	Atmospheric air
TC100*	4	Atmospheric air
Schneider*	4	Atmospheric air

From Davis, 2002.
*With Hanks's balanced salts solution.

2.5.2 Osmolality

Most cell lines present a wide tolerance range to osmotic pressure. Given the osmolality of human plasma (290 mOsm/kg), it may be reasonable to assume that this is an optimal value for human cells cultivated *in vitro*, although it can be different for cells from other species. As a general rule, the optimal osmolality range for mammalian cells in culture is from 260 to 320 mOsm/kg, while for insect cells higher values are optimal, from 340 to 390 mOsm/kg.

In laboratory and industrial practice it is important to verify the osmolality of all culture media after alterations in their basic formulation

due to the addition of salt solutions, supplements, pharmaceuticals, and hormones, or large quantities of buffering agents. Also, metabolic transformations that occur during cell culture can cause osmolality changes.

Culture medium osmolality can also increase due to evaporation, since culture flasks are generally not sealed so as to allow equilibrium between culture medium and the CO_2–air gas mixture. A slightly hypotonic culture medium can be more adequate for open cultures in multiple well plates or in Petri dishes to compensate for evaporation during incubation. To avoid large variations in osmolality during culture, the relative humidity of the culture environment should be maintained near to saturation.

2.5.3 Temperature

Temperature plays a very important role in cell culture. It has a critical influence on cell growth and it affects the solubility of various medium components, especially of gases such as CO_2 and O_2, which have low solubilities. Most mammalian cells have optimal growth rates within the range of 35–37°C. Insect cells grow at optimal temperatures of 26–28°C, while cold-blooded vertebrate cells normally grow well at lower temperatures.

2.5.4 Oxygen supply

The most important components of the gaseous phase are oxygen and carbon dioxide. Monitoring and controlling these gases in culture medium are essential for *in vitro* animal cell culture.

Cultures vary significantly with respect to oxygen demand, but in general, for most cells, oxygen partial pressure conditions slightly below atmospheric pressure are preferred. Because of that, maintenance of dissolved oxygen concentration at a range from 30 to 60% for mammalian cells is important.

Oxygen is frequently the first component to be limiting in high cell densities due to its low solubility in aqueous medium. Aeration should be performed gently and can be done by the following methods, either directly or in an isolated vessel: surface aeration, gas bubbling, diffusion through membranes, increasing O_2 partial pressure and atmospheric pressure.

For surface aeration of static cultures, in a closed system, a void volume 10 times larger than the culture medium volume is necessary for adequate oxygen supply. In agitated cultures with surface aeration, oxygen transfer is dependent on agitation rate and impeller geometry.

Gas bubbling is a simple and efficient way to transfer oxygen. Nevertheless, this procedure can cause damage to animal cells due to bubble formation. Alternatively, oxygen can be efficiently transferred to culture medium through hydrophobic membranes (silicon tubes), but this method is inconvenient, presents high cost, and is difficult to scale up, since the tube length for adequate oxygen supply in high capacity reactors needs to be very long. An increase in O_2 partial pressure also increases oxygen solubility and its diffusion rate, but it should be employed only

at high cell density, otherwise oxygen toxicity effects could occur. Although oxygen is essential for cell growth, it can be toxic in high concentrations.

Nonstatic cultures of animal cells are common with agitation and aeration performed as associated operations. These processes, allied to the fact that animal cells do not possess a cell wall, can result in cell damage if they are intense, provoking effects that alter cell metabolism, cell cycle, DNA synthesis, and protein expression, and induce cell death by apoptosis or necrosis.

For suspension cultures, cell injury can occur due to the disruption of ascending bubbles or of those associated with the foam accumulated at the surface. Many additives, such as serum and the surfactant Pluronic® F68, have been employed since the 1950s to protect cells from fluid-mechanical forces.

Serum is known as a good cell protector agent (Cartwright, 1994), and this effect is normally attributed to the increase in medium viscosity. However, since there is a strong tendency to eliminate serum from current technological applications of animal cell cultures, the use of methylcellulose and carboxymethylcellulose as serum substitutes has shown very promising results. For more complex cases, in which fluid-mechanical protection is required, as in bioreactors with intense agitation and bubbling aeration, the use of Pluronic® F68 at 0.1% (v/v) concentration can result in an efficient cell-protecting effect.

2.5.5 Composition and nature of the substratum for cell adhesion

Adherent animal cells (anchorage-dependent) need a surface for adhesion, spreading, and proliferation. Glass and plastic are the most common materials employed as a solid substratum.

For several decades, glass was the only substratum used for animal cell culture. As a consequence, the common term "*in vitro*" means "in glass." Animal cells present good adhesion on glass surfaces, especially borosilicate surfaces, that have a high silica content. Glass is still used as a solid substratum on a small scale, but very rarely in cultures aiming at high cell quantities.

The use of plastic materials for routine cell culture on the laboratory scale was introduced at the end of the 1960s, and some characteristics of glass surface, such as hydrophobicity and negative charge, were maintained in these materials. Polystyrene is the most widely used plastic material for animal cell adhesion at present, because of its surface characteristics, its low cost, and also its transparency. For more demanding cell lines, the surface has to be submitted to a treatment that involves coating with proteins such as poly-lysine, poly-ornithine, or extracellular matrix-derived proteins such as fibronectin, laminin, and collagens.

The use of microcarriers as substrates is recommended for adherent cell culture on larger scales. Microcarriers are generally spherical particles that provide a large effective area for cell adhesion, allowing adherent cell culture in homogeneous agitated systems. Microcarriers can be produced from a variety of materials such as glass, dextran, agarose, collagen, or modified polymers, and are easily recovered. The same requirements to

obtain adequate cell adhesion and growth in culture flasks are applicable. Cell growth characteristics and animal cell culture applications on microcarriers are further discussed in other chapters of this book.

2.6 Cryopreservation and storage of cell lines

Cells can be unstable when in culture for long periods, exhibiting alterations in their morphology, function, growth pattern, and karyotype. Animal cells show *in vitro* changes related to age and some cultures can suffer spontaneous transformation, showing altered growth or changes in functional characteristics. When adequately frozen, cells can be preserved for long periods without alterations in viability or in other characteristics. Thus, a cell bank, consisting of frozen and stored cell lines, makes it possible to maintain the cultures as a renewable source. This procedure enables repeated cultures to be performed with cells having equivalent characteristics, with consistent passage numbers. Cells stock also minimizes the risk of losing a culture due to accidents such as contamination by microorganisms or other cell lines, or due to failure of equipment such as CO_2 incubators.

Culture conditions influence the survival of cells submitted to cryopreservation. To be frozen, cells should be in an active growth phase, with a viability greater than 90% and free of contaminants. The optimal cryopreservation conditions are different for each cell line. When a cell is exposed to low temperatures, ice crystals are generally formed and can disrupt the cell membrane, causing death. Therefore, cells should be treated with a cryoprotector to support the freezing and thawing processes.

The optimal cell concentration in a suspension of cryoprotector medium depends on the cell type and is determined empirically, but is generally within the range of 1×10^6 to 1×10^7 cells/ml. When added to medium at concentrations between 5 and 10% (v/v), cryoprotectors such as glycerol or dimethylsulfoxide (DMSO) affect membrane permeability, allowing water release from the cell interior during cooling. Cryoprotectors decrease the freezing point, and ice crystals start to be formed at −5°C. Water efflux plays a key role in the freezing process. With cell dehydration, occurring at between −5°C and −15°C, ice crystals are formed around the cells and not inside them. The cooling velocity is also critical and should be low (about 1°C/min). Freezing conditions should minimize crystal formation in the cell interior, preventing lysis. For cells cultivated in the presence of serum, the cryopreservation medium should also contain fetal bovine serum to protect cells from the stress associated with the freezing and thawing processes. Generally, serum is added at concentrations above 20%, and can attain 90% of volume of the cryopreservation medium.

Frequently cells are stored immersed in liquid nitrogen at −196°C. However, storage in vapor phase liquid nitrogen (at −140°C to −180°C) is recommended, avoiding possible contaminations or cell death in case of cryotube rupture.

During cell thawing, the heating rate should be high. As a general practice, flasks containing frozen cells are immersed in water at 37°C, and thawing occurs in about 90 seconds. Some cells are particularly susceptible

to osmotic shock during thawing and culture medium transfer processes. Routine protocols normally describe a dilution stage (1:10 to 1:20 v/v), in which fresh culture medium should be gradually added to the thawed cell suspension. Alternatively, cells can be suspended initially in a solvent that does not permeate the cells, equimolar to the cryoprotector, which would allow its diffusion from cell membrane before transferring the cells to an isotonic medium.

For cell line storage, a cell bank is generally established, with initially three to five flasks. One of these flasks is then thawed and the cell population is expanded to produce a master bank with about 10 to 20 flasks, depending on future requirements.

As a quality control, some flasks of this bank (about two or three) should be used to confirm that the cell bank concentration and viability are satisfactory and free of contaminants. Then, a flask of the bank should be thawed and the cells cultured to obtain a working bank. The size of this bank will depend on the future demand. At this stage it is important to confirm that the master and the work banks are genetically identical.

Cell bank management insures the maintenance of the cell line original characteristics, consistency, use of cells with the same passage number, and also the availability of the cells for culture when required.

2.7 Culture quality control and laboratory safety

Cell culture practice requires rigorous control with respect to the quality of material and reagents, the origin and integrity of the cell lines, and the absence of microbial contamination.

The culture system should be totally free of compounds that can cause toxic or inhibitory effects. High concentrations of a culture medium component in culture may be growth inhibitory, even if only by increasing the osmolality. Contaminants in medium components and in the water employed for culture media formulation can also be toxic. Flasks that have not been well rinsed can expose cells to significant quantities of detergents or other components that are prejudicial for cell culture. Light (particularly short wavelength) can interact with certain culture medium components, such as riboflavin, tyrosine, and tryptophan, and generate toxic products.

Obtaining cell lines from known and safe sources is important to guarantee the use of well-characterized and authentic cells with respect to DNA profile, species of origin, and contaminants.

Microbial contamination is the most frequent problem in cell culture. The main contaminant types are bacteria, fungi, mycoplasmas, and viruses. Bacterial and fungal contamination is detected by a rapid increase in medium turbidity or by a rapid pH change. After contamination, animal cells generally survive for a short period. Mycoplasma contamination is the most difficult to detect and can cause a reduction in cell growth rate, morphological alterations, chromosomal aberrations, and changes in metabolism of amino acids and nucleic acids. Virus contamination causes changes in cell growth rate, and fetal bovine serum is usually the main source of this kind of contaminant. Some cell lines are immortalized employing virus, but these cases are not considered as contaminations.

Another problem that is underestimated in animal cell culture is the routine use of antibiotics. Continuous utilization of these compounds is not advised because it favors the development of resistant microbial strains that become difficult to eradicate, requiring the need for potent antibiotics that could be more toxic to the animal cells. Furthermore, the use of these compounds tends to mask contamination at reduced levels.

When contamination occurs, it is recommended to discard the culture and continue working with contaminant-free stocks. If this is not possible, antibiotics could be used to try to eradicate the contamination. Nevertheless, viral contaminations cannot be treated since viruses do not respond to antibiotics. Removing them by centrifugation or other separation techniques is not possible, since they are intracellular parasites. If virus-free stocks do not exist, a risk evaluation should be performed before continuing to work with the infected cell line.

A cell culture laboratory, in which activities are restricted to manipulation of established or pathogen-free derived cell lines, is a relatively safe workplace. Major risks are related to potential injuries resulting from liquid nitrogen manipulation or glassware accidents.

When developing activities involving cell lines carrying pathogens or primary cultures derived from infected animals, there is a potential risk of operator infection. Viruses present the highest contamination risk, but many bacteria, fungi, mycoplasmas and parasites can also be harmful to the operator.

Continuous cell lines not derived from humans or primates and well-characterized diploid human cells lines with a finite lifespan, for example MRC-5 cells, are considered low risk cell lines. Poorly characterized mammalian cell lines are classified as medium risk cells. Among high risk cell lines, are human and primate tissue cells, cell lines carrying endogenous pathogens, and cell lines manipulated after experimental infections.

2.8 Characteristics of the main cell lines employed industrially

The cell lines most commonly employed for production of biopharmaceuticals include CHO-K1, BHK-21, and Vero cells, besides various antibody-secreting hybridomas. Other cell lines, such as NS0, HEK-293 and PER.C6®, have also been employed for recombinant protein production.

The CHO-K1 (Chinese hamster ovary) cell line was established by Puck *et al*. (1958), and was isolated from a Chinese adult hamster. It was one of the first mammalian cell lines developed with success for utilization in recombinant protein production. The CHO-K1 cell line consists of epithelial cells that can be adapted for suspension growth. These have become the major cell type employed for recombinant protein production.

The BHK-21 (baby hamster kidney) cell line consists of adherent fibroblasts, that can also be adapted to suspension culture, and was isolated from five 1-day-old hamsters (McPherson and Stoker, 1962). These cells are commonly used for virus propagation (polio, rabies, and foot-and-mouth disease) for production of veterinary vaccines.

Vero cells, isolated from adult African green monkey kidney, consist of adherent fibroblasts that show continuous growth (Yasumara and

Kawakita, 1963). This cell line is certified by the WHO (World Health Organization) and is usually employed for virus propagation (polio, rabies) for human vaccine production. Another important application of these cells is in cytotoxicity studies of biomaterials projected for repairing or reconstituting injured human tissues.

Hybridomas, described initially by Köhler and Milstein in 1975, are immortal cells generated by the fusion of myeloma cells (immortal tumor lymphocytes that lose their capacity for producing antibodies) with normal B lymphocytes commonly obtained from rodent spleen and that have a limited life cycle before the fusion process. Among the major fusion-promoting agents employed for hybridoma production are polyethylene glycol (PEG) and Sendai virus. Procedures for obtaining hybridomas producing monoclonal antibodies (mAbs) are discussed in detail in Chapter 17.

The NS0 cell line, derived from mouse myeloma cells, is one of the most popular for heterologous proteins expression on a large scale due to its capacity for incorporating exogenous DNA and stably producing recombinant proteins. This cell line grows in suspension in a highly dispersed form, without clump formation. NS0 cells show robust growth with the production of high levels of proteins in different types of culture media. Nevertheless, when used for recombinant antibody expression, these cells may incorporate an antigenic glycan residue into the immunoglobulins that in some cases can result in adverse immunogenicity from interaction with about 1% of circulating antibodies in humans.

PER.C6® cells have been used extensively for the production of gene therapy vectors. This cell line was derived from the immortalization of human embryonic retina cells through the use of adenovirus E1 gene (Fallaux *et al.*, 1998). This cell line has been well characterized since its establishment, and no retroviruses or adventitious viruses have been detected in it. This cell line is easily adapted to different growth conditions and stably produces high levels of recombinant proteins.

HEK-293 cells, derived from human embryo kidney, were transformed with human type 5 adenovirus (Graham *et al.*, 1977). These cells exhibit epithelial morphology and can be adapted to suspension growth in serum-free media. In addition, this cell line is easily transfected and has been explored for viral vector production for gene therapy and for obtaining human recombinant proteins with normal glycosylation profiles.

The MRC-5 cell line, derived in 1966 from normal human lung tissue (Jacobs *et al.*, 1970), is adherent and shows a fibroblast morphology. These cells are well known owing to their susceptibility to several virus types, being employed in assays related to viral transfection, in cytotoxicity evaluation, and in vaccine production.

2.9 Culture of insect cells

Insect cell culture was started in 1915 but no cell line capable of replicating indefinitely was established until 1962. In that year Grace showed the ability of the insect cell line *Antheraea eucalypti* to replicate indefinitely *in*

vitro (Brooks and Kurtti, 1971). The insect cell lines most frequently cultured *in vitro* are Sf-9, High-Five™, and S2.

Sf-9 cells are derived from the IPLBSF-21 cell line, isolated by Vaughn *et al.* (1977) from pupal ovarian tissue of the fall armyworm *Spodoptera frugiperda*. This cell line is commonly used for bioproduct expression employing baculovirus as vectors, similarly to the High-Five™ cell line.

High-Five™ cells (BTI-TN-5BI-4) are derived from *Trichoplusia ni* cells and are frequently employed due to their capacity to express high protein levels when compared with other insect cell lines, such as Sf-9 cells (Rhiel *et al*., 1997). This cell line shows high growth rates in adherent culture, and can easily be adapted to grow in suspension and in serum-free media.

The S2 cell line, isolated from late stage fruit fly *Drosophila melanogaster* embryos, was established in 1969 by Schneider, from hundreds of 20–24-hour-old embryos (Schneider, 1972). This cell line can be genetically modified and is capable of stably expressing heterologous proteins, being easily adapted to suspension growth.

A comparison between insect and mammalian cell culture technology shows various similarities and differences. Due to the fact that insect cell lines typically derive from specific organs or non-differentiated embryonic tissues, the same cell line can proliferate in suspension or in adherent manner (in monolayer). This versatility in growth mode, which contrasts with most mammalian cell lines, increases the choices of bioreactor types and culture strategies for production. Contact inhibition shown by various mammalian cell lines is low or absent for insect cells, and they tend to aggregate in suspension or in adherent cultures.

Adherent insect cell release from solid surfaces generally does not require trypsinization, unlike anchorage-dependent mammalian cells. Insect cells, like mammalian cells, need rigorous aseptic manipulation during cell transfer, inoculation, and propagation in bioreactors. A minimal inoculum density is required for both cell types. Typically, insect and mammalian cell cultures are initiated with inocula of $1–2 \times 10^5$ cells per milliliter of liquid medium (Agathos, 1991). *Table 2.3* summarizes the major differences and similarities between these cells.

2.10 Use of animal cell culture in cytotoxicity assays

Besides their utilization in the production of many compounds with therapeutic, diagnostic, and immunizing applications, animal cell cultures have undoubted utility in the performance of *in vitro* cytotoxicity tests. They can be used for the evaluation of potential anti-neoplastic agents and assessment of the safety of various products, such as pharmaceuticals, cosmetics, alimentary additives, pesticides, and industrial chemical products. Cell culture systems are frequently employed in the cancer chemotherapy field, in which their potential value for viability and cytotoxicity tests is largely accepted. Animal models play an important role in toxicity testing, but the pressure to adopt *in vitro* tests is growing since they present considerable economical advantages over *in vivo* tests. The use of animal models is limited to human metabolism studies, and there are

Table 2.3 Comparison of insect and mammalian cell cultures

Characteristics	Insect cells	Mammalian cells
Cell line maintenance	Relatively easy	More difficult
Suspension and adherent versatility	Yes	No
Immortality	Yes	Only transformed cell lines
Inhibition contact	Mild or absent	Yes, except for some transformed and lymphoblastoid cell lines
Release from solid surface	Gentle agitation	Trypsinization
Sensibility to pH changes, dissolved oxygen, temperature, and osmolality	Relatively low	Relatively high
Inoculum concentration dependency to grow	Yes	Yes
Aseptic manipulation	Required	Required

Adapted from Agathos, 1991.

ethical considerations for their use in experiments (Freshney, 1992). When assays with animals are inevitable, *in vitro* cytotoxicity tests can be useful to plan *in vivo* experiments, contributing to a rational selection of types and concentrations of active agents to be tested in animals.

The evaluation of different chemicals involves: identification of potential active agents and the mechanisms by which they present their toxic effect, prediction of effective pharmaceutical cytotoxicity for treatment of patients with cancer, evaluation of the activity range of the studied compound, identification of a target cell population and of the toxic concentration range, and the relation between pharmaceutical concentration and exposure period to reach a desired activity. The chosen assay system should provide a reproducible dose-response curve with low variability over a concentration range that includes *in vivo* exposure. In addition, the selected response criterion should present a linear relationship with cell number, and the information obtained with a dose-response curve should be related to the *in vivo* effect of the same active agent or drug.

The choice of the assay to be performed depends on the context in which it will be used, the origin of the target cells, and the nature of the tested compounds. Among different assays, the parameters that vary are: cell culture method, exposure time and concentrations, recovery time after exposure, and the method used to quantify the pharmaceutical effect. This topic is briefly covered in this chapter and is discussed in detail by Freshney (2005).

2.10.1 Culture methods

The choice of cell culture method for cytotoxicity studies for a certain substance depends on the target cells and assay duration. Organ, spheroids, suspension cells, flask surface, and multi-well plate monolayers, as well as agar surface cultures can be employed.

Organ cultures allow tissue integrity and intercellular relations to be maintained, with a closer approximation to the *in vivo* situation, compared with other methods. Nevertheless, reliable quantification of the effects caused by a pharmaceutical compound is more difficult because of variability between replicates.

Spheroids are formed through spontaneous aggregation of cells, and their use allows the analysis of the effects of three-dimensional distribution of cells to test compounds, without the disadvantages presented by organ culture. Parameters such as penetration barriers in non-vascular areas and metabolic and proliferation gradients can be studied by use of this model.

Short duration cultures (4–24 hours) can be performed to evaluate the effects of pharmaceuticals on various cell types. When human tumor cells are used, the advantages include: no requirement for growth, clone minimization, presence of fast growing stromal cells, and speed in obtaining results. The disadvantage of a short duration assay is that it does not assess the effect of long exposure to the tested drug. Also, it does not consider the reversibility of the pharmacological effect or late cytotoxicity effects. Intermediate length cultures (4–7 days) are more adequate, for instance, for studies of chemical sensitivity to hematological cancer.

The monolayer culture technique is frequently employed in cytotoxicity tests with cancer lines and in studies of chemical sensitivity of different tumor types. This method offers high flexibility with respect to drug exposure and recuperation conditions, as well as to the quantification of any effect. Among all methods, cell culture in a monolayer requires a lower cell number and allows the evaluation of multiple drugs in large concentration ranges.

Although the method of clone formation in monolayer can be applied to cells directly cultivated from a tumor, cloning in suspension is more widely employed to minimize anchorage-dependent stromal cell growth. For this, cells are inoculated into soft agar after drug exposure. This assay is more adequate for cell lines that present high plating efficiencies, and is particularly suitable for solid tumors and patient effusions.

2.10.2 Exposure time and active agent concentrations

The choice of the concentration of the drug to be tested should consider therapeutic levels that could be attained with clinically employed doses. In the case of a compound under pre-clinical evaluation for a potential anti-tumor activity, a concentration limit of 100 μg/ml is recommended. Pharmacokinetic data are available for various anti-neoplastic clinically used drugs, with information about their maximum plasma concentration, concentration versus time, and pharmaceutical half-life in plasma. When these data are not available, an approximation of plasma levels could be obtained by calculating the theoretical concentration obtained when the administered dose is uniformly distributed throughout the body fluid.

Pharmacokinetic data show that maximum pharmaceutical exposure occurs generally in the first hour after intravenous injection and, for this reason, a test exposure period of 1 hour is commonly chosen. Nevertheless, this period may not be sufficient for certain cells and drug absorption

rates may be a limiting factor when short exposure periods are employed. Therefore, establishing the drug exposure period is often a question of trial and error.

2.10.3 Recovery time

The recovery period after drug exposure is important for many reasons. This period permits the re-establishment of metabolic equilibrium when inhibition is used as a pharmaceutical test. In addition, cells can recover from sublethal damage or indicate effects of late cytotoxicity, which are not revealed in alternative tests.

Depending on the nature of the drug and on the method of evaluation, the measurement of a recovery period could result in underestimation or overestimation of any lethal effect on cells. It is important that the recovery period is not very long, since cell death could be masked by a resistant population growth.

2.10.4 Cytotoxicity evaluation methods

Cytotoxicity assays can be performed in various ways. In some cases, alterations of metabolic activities induced by drugs are measured. In other cases, structural integrity, which may or may not be directly related to cell death, is evaluated. Alternatively, cell survival assays measuring the result of each metabolic perturbation can be performed and used to evaluate cell recovery or death.

Theoretically, the only conclusive signal of cell survival after drug exposure is the demonstration of reproductive integrity, which can be evaluated through plating efficiency and cell proliferation tests. Nevertheless, metabolic parameters could also be employed as a survival measure when the cell population is submitted to a recovery period after drug exposure.

Some cytotoxicity assays, such as dye incorporation by dead cells or ^{51}Cr or fluorescein release by labeled cells, offer instantaneous results and are named viability tests. These tests are adequate for the identification of dead cells, but can overestimate cell survival over longer periods. Most of them cause membrane rupture and cell death.

Other cytotoxicity assays, such as those based on the assessment of metabolic events, can be more reliable and sensitive. The assessment of viability is less reliable since many forms of metabolic inhibition can be reversible.

Evaluation of membrane integrity is the most commonly used measurement of cell viability, indicating instantaneous or progressive damage over a few hours, and can be performed by ^{51}Cr or specific enzymes release or a dye exclusion assay. These assays are particularly important for toxic agents that exert a primary effect on membrane integrity. Nevertheless, quantification can be difficult due to cell loss by surface detachment or autolysis.

Measurements of changes in respiration (O_2 utilization) induced by drugs or in glycolysis (CO_2 production), as well as changes of dehydro-

genase activity or pH can also be used to evaluate the cytotoxicity of certain compounds.

The measurement of incorporation of radioactive metabolites is frequently used as a response to short and intermediate duration cytotoxicity. Measurements of [^{3}H]-thymidine incorporation into DNA and [^{3}H]-uridine incorporation into RNA are the two most common methods of quantifying drug cytotoxicity. Furthermore, [^{125}I]-iododesoxyuridine, a specific and stable label for DNA synthesis, is also employed, as well as measurements of [^{32}P]-phosphate release into medium or incorporation into nucleotides, in addition to the incorporation of [^{14}C]-glucose, [^{3}H]-amino acid, and ^{45}calcium.

Determination of protein content is a relatively simple method to estimate cell number. Cytotoxicity can be evaluated by this method from an alteration in protein accumulation over the time of culture.

In addition, there are several tests that quantify cell number through a color development in the medium, including assays based on protein content, DNA content, lysosome and Golgi complex activity, enzymatic activity, and MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H tetrazolium bromide) dye reduction.

References

Agathos SN (1991), Production scale insect cell culture, Biotechnol. Adv. 9:51 68.

Augusto EFP, Oliveira MS (2001), Processos com Células Animais, In: Lima UA, Aquarone E, Borzani W, Schmidell W (Eds), Biotecnologia Industrial, vol. 3, Processos Fermentativos e Enzimáticos, Edgard Blüncher, São Paulo, pp. 547 582.

Brooks MA, Kurtti TJ (1971), Insect cell and tissue culture, Annu. Rev. Entomol. 16:27 52.

Cartwright T (1994), Animal Cells as Bioreactors, Cambridge University Press, New York.

Davis JM (2002), Basic Cell Culture. A practical approach, 2nd ed., Oxford University Press, New York.

ECACC Handbook (2005), Fundamental Techniques in Cell Culture. A Laboratory Handbook. Available in http://www.sigmaaldrich.com/Area of Interest/Life Science/Cell Culture/Key Resources/ECACC Handbook.html (accessed May 2005).

Fallaux FJ, Bout A, van der Wollenberg DJ, Hehir KM, Keegan J, Auger C, Cramer SJ, van Ormondt H, van der Eb AJ, Valerio D, Hoeben RC (1998), New helper cells and matched early region 1 deleted adenovirus vectors prevent generation of replication competent adenoviruses, Hum. Gene Ther. 9:1909 1917.

Freshney RI (1992), Animal Cell Culture A Practical Approach, 2nd ed., IRL Press, Oxford.

Freshney RI (2000), Culture of Animal Cells. A Manual of Basic Technique, 4th ed., John Wiley & Sons, Inc., New York.

Freshney RI (2005), Culture of Animal Cells. A Manual of Basic Technique, 5th ed., John Wiley & Sons, Inc., Hoboken.

Graham FL, Smiley J, Russel WC, Nairn RJ (1977), Characteristic of a human cell line transformed by DNA from human adenovirus type 5. Gen. Virol. 36:59 72.

Hayflick L, Moorhead PS (1961), The serial cultivation of human diploid cell strains, Exp. Cell Res. 25:585 621.

Jacobs JP, Jones CM, Baille JP (1970), Characteristics of a human diploid cell designated MRC 5, Nature, 227:168 170.
Kilburn J (1991), Monitoring and control of bioreactors, In: Mammalian Cell Biotech nology: A Practical Approach, Butler M (Ed.), Oxford University Press, New York, pp. 159 184.
Kohler G, Milstein C (1975), Continuous cultures of fused cells secreting antibody of predefined specificity, Nature 256:495 497.
Macpherson I, Stoker M (1962), Polyoma transformation of hamster cell clones an investigation of genetic factors affecting cell competence. Virology 16:147 151, 1962.
Puck TT, Cieciura SJ, Robinson A (1958), Genetics of somatic mammalian cells. III. Long term cultivation of euploid cells from human and animal subjects, J. Exp. Med. 108:945 956.
Rheinwald JG (1989), Methods for clonal growth and serial cultivation of normal human epidermal keratinocytes and mesothelial cells, In: Baserga R (Ed.), Cell Growth and Division: A Practical Approach, IRL Press, Oxford, pp. 81 94.
Rhiel M, Mitchell Logean CM, Murhammer DW (1997), Comparison of *Trichoplusia ni* BTI Tn 5B1 4 (High Five™) and *Spodoptera frugiperda* Sf 9 insect cell line metabolism in suspension cultures, Biotechnol. Bioeng. 55:909 920.
Schneider I (1972), Cell lines derived from late embryonic stages of *Drosophila melanogaster*, J. Embryol. Exp. Morphol. 27:353 365.
Vaughn JL, Goodwin RH, Tompkins GJ, McCawley P (1977), The establishment of two cell lines from the insect *Spodoptera frugiperda (Lepidoptera; Noctuidae),* In Vitro 13:213 217.
Yasumura Y, Kawakita M (1963), The research for the SV40 by means of tissue culture technique, Nippon Rinsho 21:1201 1219.

Cloning and expression of heterologous proteins in animal cells

3

Mariela Bollati-Fogolín and Marcelo A. Comini

3.1 Introduction

Among the eukaryotic organisms, the animal cells are consolidated as basic tools for the production of proteins with therapeutic applications. The steady progress in the design and generation of novel expression vectors and cell lines, the remarkable improvements in the composition of the culture medium and in the area of process control, made feasible the production of recombinant proteins on a large scale. One of the pivotal factors that determines the success of the first phase in a biotechnological process lies in the correct selection of the expression vector, host cell, and culture medium. Moreover, the genetic manipulation of the first two and the choice of the appropriate method for transfection and genetic amplification may influence positively the specific cellular productivity. In this respect, this chapter presents the basic fundamentals and the practical concepts that have to be considered in the initial stage – cloning and expression – of a biotechnological process based on animal cells.

3.2 The flow of genetic information and molecular cloning

In order to express a gene in a eukaryotic cell, it is not enough just to have the sequence of deoxyribonucleic acid (DNA) encoding the polypeptide of interest. The presence of sequences that regulate gene expression is essential. A typical eukaryotic transcription unit contains the information that governs the transcription rate of a gene, namely the speed at which this DNA sequence is copied to ribonucleic acid (RNA). This primary transcript or immature RNA carries the signals that determine the rate and nature of its processing into a mature messenger RNA (mRNA). In the cytoplasm, the codons of the mRNA are translated into an amino acid sequence that gives rise to a polypeptide. This amino acid sequence dictates the three-dimensional structure, stability, function, cellular localization, post-translational processing (i.e. proteolytic cleavage, glycosylation, etc.), and/or the secretion of the new protein.

The first step towards the heterologous expression of a gene consists in the isolation of its sequence either from genomic DNA (gDNA) or mRNA. The sequence isolated from gDNA, in most of the cases, contains

the regulatory elements that control its expression (see below), while that obtained from mRNA lacks many of them and requires their incorporation to enable an efficient transcription-translation. Although a sequence isolated from gDNA can be transferred directly to the host cell, the success of its expression can be limited significantly because the regulatory sequences may not be recognized by the transcriptional/translational machinery of the host. gDNA sequences are large and this makes their manipulation difficult and considerably reduces the efficiency of transfection. Such problems are generally overcome by cloning the exogenous gene into, and expressing it from a vector (plasmid, virus) that provides the elements controlling the rate of generation, and the processing of the transcript. For details about molecular biology methods and recombinant DNA technology, Sambrook *et al.* (2000) is recommended.

3.3 Elements required for gene expression in eukaryotic cells

In general, any animal cell is able to express foreign genes as long as those genes contain regulatory elements that can be functional in the host cell. A typical eukaryotic transcription unit consists of a gene flanked by upstream and downstream sequences. These flanking regions encode the information that dictates the rate of transcription, the processing, and the stability of the mRNA, as well as other properties of the polypeptide. The sequence of a gene can exceptionally contain elements that may influence its transcription-translation. The most relevant features of these elements are described below and have been classified into two groups according to the level at which they operate, transcriptional or translational, respectively.

3.3.1 Transcriptional control elements

In eukaryotic organisms, transcription regulation is a complex process that demands coordinated interaction of several genetic elements. The efficiency of this process mainly depends on the promoter/enhancer sequences, the copy number of the gene, and the structure and elements present at the insertion site in the host′s chromatin. On the other hand, the co-transcriptional modifications (capping, splicing, polyadenylation, and transport to cytoplasm) on the primary transcript determine the stability, turnover rate, and translational capacity of the future mRNA.

Promoter, enhancer, and chromosomal elements

The promoter is the set of sequences located upstream from the beginning of the transcription, whose function is to regulate the rate limiting step of this process: the initiation of the transcription of the adjacent gene. The basic sequence of all promoters consists of the TATA-box, a region highly conserved that serves as an anchorage signal for the complex that will start the transcription. Other sequences that activate the transcription are the CCAAT- and GC-box, which are located about 40–110 bp upstream from the TATA-box. Depending on the promoter, additional sequences can

determine the type and specificity of the transcriptional activity (Novina and Roy, 1996). For instance, there are promoters that carry out the transcription in a constitutive, inducible, or tissue-specific mode, or that are activated in particular stages of the cell development or cell cycle (see Section 3.4.3).

The activity of the promoter can additionally be increased by enhancers. These elements increase the rate of transcription in a manner that is independent of their orientation, position, and distance with respect to the gene to be expressed (Fromm and Berg, 1983). The enhancers have been suggested to bind the transcriptional complex at the promoter sequence, facilitating the transcription by altering the local structure of the chromatin and/or stabilizing the complex (Szutorisz *et al.*, 2005). Unlike promoters, enhancers do not present a conserved sequence, and hence, it is difficult to predict if a certain transcription unit contains them or not. Although enhancers do not show specificity for promoter sequences, their activity is constrained to certain cellular types or developmental stages (Szutorisz *et al.*, 2005). Specific mutations introduced into the enhancer sequences allow an increase in their potency and the number of cell lines where they are active (Yaniv, 1982).

Certain structural domains of the chromatin, designated scaffold-attached-regions (SAR), locus-control-regions (LCR) and insulators, that are intrinsically associated with transcriptionally active regions of the genome, have recently been discovered. These elements induce the unfolding of the heterochromatin (transcriptionally inactive) and the separation of the DNA chains assisted by proteins of the internal matrix of the cellular nucleus. Thus, the RNA-polymerase and other transcriptional factors can gain access to this locus and, consequently, activate the expression of the neighboring genes (Hauser, 1997). In contrast to SARs, the activity of LCRs is cell-specific (Klehr *et al.*, 1991). The insulators isolate loci by means of a steric effect (i.e. formation of hairpin loops) inhibiting the recruitment of the enzyme histone-acetyltransferase and thus, preventing the activation or repression of certain specific chromosomal regions (Gerasimova and Corces, 2001; West *et al.*, 2002). The inclusion of these elements in expression vectors represents a novel strategy to avoid the positioning effect, common when vectors for chromosomal integration are used (discussed in Section 3.4.3).

Capping, introns, polyadenylation, and transcription-termination sequences

Capping is a co-transcriptional process that consists of the addition of 7-methylguanosine to the 5′ mRNA end, which protects the transcript from degradation and allows its further processing. In general, the promoter sequence provides the capping signal. The removal of the 5′ methylguanosine and the 3′ structures from the RNA constitute the critical steps that determine the half-life of the transcript.

Introns are non-coding sequences that are excised from the precursor mRNA by specific cleavage in a process known as splicing. It has been postulated that the presence of introns enhances the efficiency of transcription by stabilizing the RNA structure, and that splicing increases the

rate of polyadenylation and the nuclear export of the mRNA (Mattaj, 1990). The sequence of the introns is not conserved and the bases GU and AG in the 5′ and 3′ ends, respectively, are recognized as hallmarks of almost all introns (Petitclerc *et al.*, 1995). Although there are genes that are efficiently expressed without the presence of introns, their inclusion in expression vectors is generally recommended. They should be placed at the 5′ end of the transcription unit to avoid aberrant splicing as a result of inactive or cryptic splicing sites present in the gene sequence (Huang and Gorman, 1990).

Most mRNAs from eukaryotic cells contain in their 3′ end an extension of approximately 200 adenosine molecules, known as the polyA tail. Similar to capping and splicing, the polyadenylation of the primary transcript is a co-transcriptional process. It occurs at the 3′ end of the RNA and consists of a site-specific cleavage followed by polymerization (adenylation). The polyadenylation is essential for the formation, stability, and translatability of the mRNAs. The polyadenylation signal is composed of a highly conserved sequence (AATAAA) located about 10–30 nucleotides downstream from the stop codon, and positioned immediately upstream of a T- or TG-rich region (T/G box; Munroe and Jacobson, 1990). In most cases, the initial step in mRNA degradation is the shortening of the polyA tail. This gradual aging of the mRNA takes place in the cytoplasm and is referred to as the 'molecular clock' of the RNA. The polyadenylation signals more commonly employed in expression vectors are those derived from the bovine growth hormone, mouse β-globin, SV-40 early transcription unit, thymidine kinase (TK), herpes simplex virus (HSV), and hepatitis B antigen (Kaufman, 1990).

The sequence signaling the culmination of the transcription reaction is localized between the polyadenylation signal and the 3′ end of the mature RNA. Although the transcription termination region of several eukaryotic genes has been identified – consensus sequence: ATCAAA(A/T)TAGGA AGA – the precise site of the termination is, in most of the cases, unknown. The termination signal is required to prevent the transcriptional machinery extending its activity to sequences located downstream of the gene that is being transcribed, a phenomenon known as 'transcriptional interference.' It has also been proposed that the introduction of termination sequences abolishes or diminishes the transcription of the complementary DNA chain, which would give rise to the formation of antisense RNA, with the consequent suppression of the expression of the sense RNA (Izant and Weintraub, 1985). The expression vectors for animal cells harbor the well-characterized transcription terminator sequences from prokaryotes.

3.3.2 Translational control elements

In the cytoplasm the codons of mRNA are translated into amino acids via the concerted action of the ribosomes, transfer RNA (tRNA), and a large protein complex. In addition to the mature RNA sequence (capped and polyadenylated) other structural elements located in the 5′ and 3′ untranslated regions (UTRs) of the mRNA may influence its translation.

5′ Untranslated region

The initiation of the translation constitutes the rate limiting step in the synthesis of proteins. The start codon (**AUG**) embedded in the consensus sequence GCC(A/G)CC**AUG**G, widely known as Kozak′s sequence, gives the appropriate context for an optimal beginning of the translation (Kozak, 2005). Specific purines in the Kozak′s sequence (underlined) provide an enhancer effect on translation. It has also been noted that the existence of additional start codons in the 5′ UTR impairs severely the translation (Grens and Scheffler, 1990). The formation of secondary structures (i.e. hairpin loops) in this region may inhibit the binding and/or the advance of the translational complex on the RNA strand due to a steric hindrance. Regions rich in G and C are prone to form structures of this type, which are thermodynamically very stable (Grens and Scheffler, 1990).

3′ Untranslated region

In mammalian cells, the degradation of the mRNA is triggered after a significant shortening of the polyA tail and is catalyzed by 3′-5′ exoribonuclease activity of a protein complex termed the exosome. Mechanisms involving exonuclease activity that are independent of the polyA ‘‘aging’’ have also been described (Inácio and Liebhaber, 2003). In the 3′ UTR of the mRNA there are sequences able to alter the stability of the transcript. For instance, motifs that have AU-rich elements (ARE) were found in highly unstable mRNA. AREs were later shown to bind proteins with the capacity to regulate either positively or negatively the decay rate of the mRNA, that is, recruiting or blocking the exosome (Gray and Wickens, 1998).

Termination codon and codon usage

The completion of the translational process requires the binding of the ‘‘release factor’’ to the stop codon, which induces the disassociation of the translational complex and thus, the end of polypeptide synthesis. Although the presence of the stop codon is sufficient to interrupt the translation, exhaustive studies have demonstrated that the contiguous base can influence significantly the efficiency of this process. For example, it has been reported that purines (A or G) occupying this position constitute a much more effective termination signal than pyrimidines (C or U; McCaughan *et al.*, 1995). Two models were proposed to explain the effect of the fourth base on the performance of the termination process: one suggests that this base is recognized as a part of the stop codon and the other claims that this base constitutes an independent signal for the recruitment of the ‘‘release factor’’ (McCaughan *et al.*, 1995).

It is worthwhile to mention that a considerable degree of preference for certain codons has been observed for genes displaying high expression levels, which correlated with a higher abundance of the complementary tRNA in the cell (Fedorov *et al.*, 2002). The optimization of codon usage

can be achieved by site-directed mutagenesis to increase the expression level of some genes (Makrides, 1999).

3.4 Systems for heterologous expression in animal cells

All expression systems consist of a vector and a host. The vector is defined as a molecule of DNA or RNA, which is genetically manipulated to carry a molecule of foreign DNA or RNA, with the aim of producing proteins (gene expression) or DNA (amplification, replication) once it is introduced into a host cell. In the nucleus of the transfected cell the expression vector can exist: (i) as an independent replication unit, namely episome, or (ii) integrated to the host genome through a random or non-homologous recombination process. In order to exist as an episome the vector must have a signal (episomal replication origin) allowing for its autonomous multiplication, non-linked to the replication of the host genome. Episomal vectors render high transient expression levels of recombinant protein (discussed below). However, the cytotoxicity generated by the high concentration of exogenous DNA, the tendency to integrate into the cellular genome and to undergo rearrangements (mainly in long-term cultures) have been pointed out as the major obstacles inherent to its use (Sanders, 1990). As explained below, the clonal variation due to the ''positioning effect,'' commonly observed for integrative vectors, does not occur with episomal vectors.

The integration of the vector into the host's genome takes place in a random manner and the sequences/structures from either the vector or the site of integration can affect the expression of the target gene. The fact that nearly 95% of the genome from animal species consists of non-coding sequences and that the coding regions are frequently silenced (heterochromatin) explains the low efficiency in obtaining highly productive clones from vectors that integrate into the chromosomes. Possible rearrangements of the vector′s DNA giving rise to mutations, insertions or deletions that can alter the sequence or expression of the heterologous gene, may take place when using this type of vector.

The expression systems available for the production of recombinant proteins in animal cells can be classified as mammalian cell/viral or plasmid vector, or insect cell/baculovirus.

3.4.1 Viral vectors

The first attempts to express foreign genes in mammalian cells were carried out using viruses as vectors (Goff and Berg, 1976; Hamer *et al.*, 1977). According to the nature of the viral nucleic acid, these vectors are classified as DNA or RNA viruses, or chimeras. If the genetic material of the virus is surrounded by a protein envelope (capsid), it enters the cell by a mechanism called infection, which generally occurs with high efficiency. If the nucleic acid of the vector lacks a capsid (''naked''), its incorporation to the cell is carried out by means of transfection techniques (discussed in Section 3.7). In a permissive cell, a viral vector develops a lytic cycle, in which the replication of the viral DNA and its

later encapsulation generates a large number of virions (progeny) that will finally cause cell lysis and death, thus precluding the establishment of a stable cell line. Such limitations can be overcome by using: (i) nonpermissive cells, wherein the virus replicates as an independent entity integrated into the host's genome and induces only a morphological transformation of the cells; (ii) defective viruses, whose genome has been modified in such a way that it cannot complete the lytic cycle and depends for its propagation on either a helper virus or a specific cell line that provides the absent elements/functions (i.e. COS cells/SV-40 virus, Section 3.6.1); or (iii) genetically modified permissive cells that allow the episomal replication of the virus, increasing the productive yield while preventing the lytic cycle, although the high concentrations of extrachromosomal DNA can be cytotoxic (Sanders, 1990). The main advantage offered by the viral systems is that tedious screening for high producing clones is not required as is the case when using integrative plasmid vectors. Some disadvantages displayed by viral vectors are: (i) the physical limitations of the viral packaging restrict the size of the foreign gene; (ii) recombinant viruses are generally defective and must be propagated with a helper virus or a specific cell line; (iii) stable recombinant cell lines cannot be established with a virus that presents a lytic cycle; (iv) the specificity of the virus to infect certain cell types restrains the selection of the expression host.

Vectors derived from DNA viruses

SV-40 and polyoma viruses belong to the group of the **papovavirus** (Family: Polyoma virus), both presenting a small ''naked'' genome (~5 kb) that is able to replicate in primate and murine cells, respectively. SV-40 is considered the pioneer vector in terms of heterologous expression in mammalian cells (Goff and Berg, 1976; Hamer *et al.*, 1977), and is preferred over polyoma viruses for production of recombinant proteins due to its higher replication rate and infection efficiency. The target gene is inserted in the early or late replication regions of the viral genome, producing a recombinant defective virus (Muller *et al.*, 1983; Zhu *et al.*, 1984). The advantages of employing SV-40 as an expression vector can be summarized in the higher expression levels and short time required to evaluate the production of recombinant protein. The drawbacks of this expression system are: (i) the DNA sequence to be inserted has a maximum size of 2.5 kb; (ii) the host selection is limited because the viral infection is cell-specific; and (iii) successful recombinant expression is variable and unpredictable (Levinson, 1990).

Adenoviruses possess a genome of about 35 kb, which allows insertion of a larger heterologous sequence without affecting viral replication. This expression system offers high expression levels since viral infection inhibits synthesis of cellular proteins and, in the case of type-2 adenoviruses, they allow the use of a wide range of host cells (i.e. human, primate, rodent). Owing to the reduced number of restriction sites available in the adenoviral genome, insertion of foreign sequences is performed by homologous recombination instead of molecular cloning. In this respect, the viral DNA is co-transfected with a plasmid containing the gene of interest

flanked by the regions of the viral genome where recombination will take place. The frequency of recombination is variable and the generation of stocks of recombinant virus requires purification of the viral progeny. Sequences up to 8 kb can be inserted into and expressed by adenoviruses. Heterologous promoters (from SV-40 or cytomegalovirus) have been successfully used to express genes with this system (Sandig *et al.*, 1997). The cell line HEK-293 has been generated to complement a region absent in recombinant adenoviruses, thus obviating the need for a helper virus to complete the viral cycle. The adenoviruses are reliable vectors for the production of recombinant proteins. The generation of novel adenoviral vectors is described in detail by Bourbeau *et al.* (2003).

The **adeno-associated viruses** (AAVs) belong to the Parvovirus family, contain a small genome (5 kb), and are not pathogenic. AAVs have a biphasic life cycle in the host cell. They can either integrate to the host genome or persist in an episomal form, both mechanisms leading to a latent infection in the absence of a helper virus. In the presence of a helper virus (i.e. adenovirus or herpesvirus), AAVs undergo a productive infection. In addition to the lack of pathogenicity, these vectors present other advantages such as high transfection efficiency, reduced cytotoxicity at elevated multiplicity of infection, and a wide range of hosts. The main limitation of these vectors lies in the size of the gene that can be expressed (< 5 kb). Although the rate of integration of AAVs to the host genome is low, undesirable chromosomal mutations can be expected when employing the helper virus approach (Xiao, 2003). The recent discovery of the minimal set of adenoviral genes required for efficient generation of progeny AAV particles (Matsushita *et al.*, 1998) allows the production of recombinant AAVs without the need of adenoviral co-infection.

Epstein–Barr virus (EBV) is a member of the herpesvirus family that became useful as an expression vector for mammalian cells. A peculiarity of this virus is that it can develop either a latent infection or a lytic cycle in the host. The first leads to transformation/immortalization of the host cell, and the second to cell lysis. The identification of the elements necessary for EBV replication extends the range of permissive cells from human B lymphocytes to fibroblastic and epithelial cells of human, primate, and canine origin (Sugden *et al.*, 1985; Lutfalla *et al.*, 1989). The use of this virus as an expression vector offers stable transfection and high levels of gene expression, simplicity for the selection of highly producing clones, easy recovery of the episomal DNA, and the possibility to produce authentic human glycoproteins if human cells are used as the host (Teshigawara and Katsura, 1992). Some disadvantages of the EBV-based expression systems are the need for a selection marker to assure the persistence of the DNA in an extrachromosomal form, a certain degree of variability in the expression levels depending on the cell type, and the probability of genomic integration with a consequent reduction in the expression rate of the heterologous gene (Levinson, 1990).

Another member of this family is HSV (herpes simplex virus). HSV has a genome size of 125 kb, although half of the genes are not essential for growth, allowing large molecular inserts. HSVs infect a wide range of hosts and show a high infectivity. The relative simplicity of preparing large

stocks of recombinant virus is another advantage presented by HSV (Burton *et al.*, 2003).

Papillomaviruses contain a genome of ~8 kb, usually have a single host and replicate episomally. Bovine papillomavirus (BPV) is a representative of this family, which induces phenotypic changes in the host facilitating the identification and selection of transformants. Although BPV-based vectors replicate episomally, the selection pressure achieved by the addition of selection markers (i.e. neomycin or cadmium; see Section 3.8) has been reported to induce a stable integration of multiple copies of the viral DNA into the host genome (Niwa *et al.*, 1991). The susceptibility of the virus to undergo rearrangements with the concomitant risk of altering the sequence of interest is perhaps its main drawback (Levinson, 1990). The optimization of the expression system based on papillomavirus has been thwarted by the lack of progress in understanding the biology of the viral replication and transcription.

Vaccinia virus is a representative of the Poxviruses. Members of this family are unique among vertebrate viruses because they possess their own transcription machinery encoded in a genome of 100–300 kb. The fact that the host metabolism is dispensable allows vaccinia to have a high rate of replication and protein biosynthesis, making it an excellent choice for the expression of heterologous genes (Moss, 1996). Mammalian and avian cells are suitable hosts for vaccinia. The viral genome can accept DNA fragments up to ~25 kb, which are incorporated by homologous recombination, where in general, the gene encoding for viral thymidine kinase (TK) is replaced and selection of recombinant virus is carried out in TK-deficient cells (Mackett *et al.*, 1982). It is worthwhile to note that the transcription mechanism of vaccinia is unable to perform splicing; therefore, this vector allows only the expression of complementary DNA (cDNA) sequences (Moss, 1996). Poxvirus-based systems are commonly used in eukaryotic cells due to the stability of the viral genome, the wide range of hosts, the ease of production and manipulation of the virus, and the high expression levels (Carroll and Kovacs, 2003).

Vectors derived from RNA viruses

In **retroviruses**, the genetic information is encoded in the form of RNA (5–10 kb), which is retro-transcribed to DNA in the host cell by means of a virus-specific reversed transcriptase enzyme. Since this intermediate DNA cannot integrate into the genome of the infected cell, retroviruses are employed as transference and expression vectors (Holmes-Son *et al.*, 2001). Several cell lines that enable the production of defective recombinant retroviruses have been generated to circumvent the use of helper virus (Cone and Mulligan, 1984). The use of retrovirus as an expression vector presents the following advantages: (i) it allows the expression of genes in different cell types and species; (ii) stable recombinant cell lines can be obtained since the genetic material of the virus integrates reliably and stably in the host genome, and the viral infection does not produce cell death; (iii) it allows increased infectivity and higher viral titers; (iv) it allows high efficiency for introduction of foreign genes in animal cells when compared with the commonly used transfection methods. Two

relevant points should be considered before employing retroviral vectors: (i) although they can express genomic sequences containing introns, the progeny will not harbor them, only copies of the cDNA; (ii) polyadenylation signals in the target sequence must be avoided because they induce a premature polyadenylation and the consequent absence of full mRNAs (Shimotohno and Temin, 1981). The acceptable yields obtained with retroviral vectors are explained either by their capacity to integrate into transcriptionally active loci or, once inserted, they trigger the activation of the loci (Kaufman, 1990). However, in terms of biotechnological applications, retroviruses can hardly compete with other viral vectors and present the following limitations: the size of the foreign DNA to be expressed ($< 6–7$ kb), the moderate and variable expression level (one or few copies of the retrovirus integrate into the chromosomes), and the incapacity to co-express two heterologous sequences from a single retrovirus due to epigenic effects (Emerman and Temin, 1984). Parolín and Palú (2003) have provided an excellent review on recent progress in the design and applications of retroviral vectors.

Within the family of Togavirus, two members of the genus **Alfavirus**, namely Sindbis virus and Semliki Forest virus, stand out for their use in biotechnology. They are very simple RNA viruses with a genome encoding for replication signals, structural proteins and for an RNA-dependent polymerase. The main expression product of the infected cells is the heterologous gene, since the viral infection down-regulates the synthesis of host proteins (Strauss and Strauss, 1994). The generation and manipulation of these vectors is simple, and well-established methods exist for the production of recombinant proteins in different cell lines on a large scale (Blasey *et al.*, 1997). Lundstrom (2003) has recently outlined the applications of alfavirus as expression systems.

Among the single-strand RNA viruses, **coronaviruses** present the largest genome allowing the insertion of large heterologous sequences ($\sim$27 kb). The virus replicates in the cellular cytoplasm and does not undergo an intermediate stage as DNA. Therefore, its integration into the host genome is an unlikely phenomenon. A description of the different strategies available nowadays for the generation and use of coronaviruses as expression vectors has been published by Enjuanes *et al.* (2003).

3.4.2 Baculoviruses

Baculoviruses encompass a family of viruses that infect different species of arthropod insects. The name derives from the fact that the DNA genome, with a size between 80 and 200 kb, is packed in nucleocapsids that acquire the shape of a walking cane or *baculo*. A schematic representation of the life cycle of the virus is shown in *Figure 3.1*. The polyhedrin gene is not essential for virus growth in cell cultures and, if deleted, extracellular viral particles (ECVs) will be released from the cells. This constitutes the basis for the use of baculovirus as an expression system: the polyhedrin gene is replaced by the sequence of interest, whose transcription will, thus, be under the control of the potent polyhedrin promoter. The recombinant expression unit is transferred by homologous recombination to the locus of the polyhedrin gene of a wild-type virus.

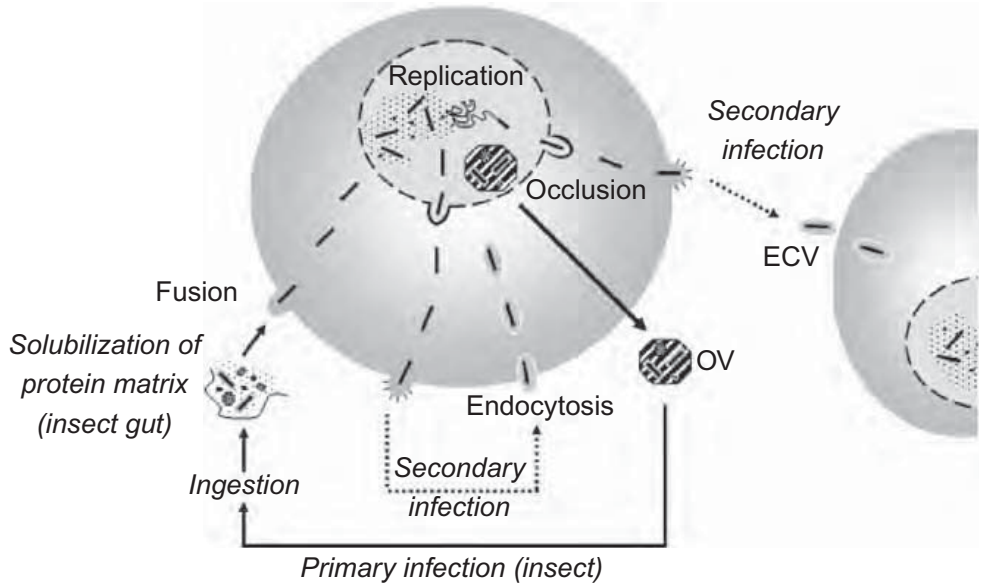


Figure 3.1

Life cycle of baculoviruses. The infection of insect cells with baculovirus produces two types of progeny, the extracellular (ECV) and occluded (OV) viral particles, which differ structurally and functionally. The nucleocapsid of ECVs is surrounded by a membrane envelope and these particles cause *in vitro* cell to cell infection but are not infective to insects. In contrast, the nucleocapsid of OVs is coated by a protein matrix, formed by polyhedrin or granulin, for the nuclear polyhedrosis or granulosis virus, respectively. OVs are responsible for *de novo* infection of insects but cannot infect cells cultivated *in vitro*. The matrix protects the particles against environmental agents (e.g. UV light, drying), but is degraded at the alkaline pH of the insect gut. The removal of the protein coat enables the infection of the gut cells by the virus, which migrates to the nucleus to accomplish the replication and transcription of its genetic material. At this stage the two viral progenies can be formed from the nucleocapsids: those accumulated in the nucleus and assembled to the polyhedrin or granulin give origin to OVs, while those emerging from the nucleus and cell membrane are ECVs.

There are three different approaches to insert genes in a baculovirus genome: (i) by homologous recombination; (ii) by site-specific transposition to a bacmid (a modified *Escherichia coli* that contains a copy of the baculovirus genome) mediated by the transposon 7 (Tn7); or (iii) by molecular cloning (Condreay and Kost, 2003). Transcriptional promoters that operate in insect cells are not active in mammalian cells; therefore, baculovirus-based expression can only be achieved by replacing the polyhedrin promoter with CMV or SV-40 promoter sequences (pCMV, pSV-40). These modified vectors display an extensive diversity of hosts (hematopoietic cells excepted) but an expression level that is cell line-dependent. Variability of expression is, at least partly, associated with transcriptional silencing of the expression cassette as a result of its incorporation into the nucleosomes in the genomic DNA (Condreay *et al.*, 1999). Besides its application for the transient expression of recombinant proteins, it is also possible to obtain stable cell lines by adding to the vector a cassette with a biochemical marker (Merrihew *et al.*, 2001). The baculovirus system offers: (i) versatility and diversity of hosts; (ii) absence

of cytotoxicity; (iii) transient or stable gene expression; (iv) integration as a single copy to the genome; (v) easy manipulation and scaling-up of the production; (vi) stability and conservation of the viral particles; (vii) capacity to accept large inserts (~40 kb); and (viii) high biosafety since baculovirus is only able to replicate in invertebrates (King and Possee, 1992). The main limitations of this system are related to the use of insect cells as a platform for the production of recombinant proteins. In this respect, the expression is discontinuous (batch), because of cell lysis/death, and the glycosylation pattern is simpler than that of mammalian cells (with relatively unbranched sugar chains) and with a high content of mannose. This is usually undesirable for some proteins with therapeutic application (see Chapter 6 and Luckow, 1995).

3.4.3 Plasmid vectors

The discovery and functional characterization of several of the elements that control the expression of genes in eukaryotic cells and viruses, in addition to progress in the field of genetic engineering, allowed the generation of plasmid expression vectors. These types of vectors consist of naked DNA with a size between 2 and 20 kb. The genetic elements they contain dictate whether they will exist as episomes or integrated into the host genome. The basic structure of the plasmid employed for expression in animal cells is made up of three cassettes: (i) an eukaryotic expression unit; (ii) a biochemical marker; and (iii) a prokaryotic replication unit (see *Figure 3.2*). The first cassette drives the expression of the exogenous gene. The biochemical marker allows for selection of clones that have stably integrated the plasmid DNA into their genome (see Section 3.8). In this cassette, the expression of the selection gene is most preferably dictated by a weak promoter to increase the chances of isolating high producer cell lines (Wurm, 2004). All the genetic manipulations required during the construction of the recombinant plasmids, as well as their amplification (replication) in sufficient amounts for future transfections, are routinely carried out in *E. coli*. Thus, unlike the viral vectors, plasmids also contain sequences controlling their replication and selection in a prokaryotic host. Some novel elements of plasmids, which have extended significantly the range of applications in the biotechnology area, are discussed below.

Inducible and specific promoters. The expression of a heterologous gene from a constitutive promoter is not recommended if the recombinant product is cytotoxic or affects cell growth. In such a case, it will be necessary to regulate the expression levels that can be achieved by means of an inducible promoter. An ideal inducible system should meet the following requirements. (i) Specificity: unresponsive to endogenous activators and absence of interference with the host physiology. (ii) Efficiency: null to low expression levels in the non-induced state, and quick response to activation or induction. (iii) Dose dependency: homogenous regulation of the expression levels. Several sequences and ligands that regulate positively or negatively the transcriptional promoters of a large number of animal cells have been identified and incorporated into expression vectors. These elements can respond to different external stimuli such as heat shock, hormones, heavy metals, cytokines, or hypoxia. However, these

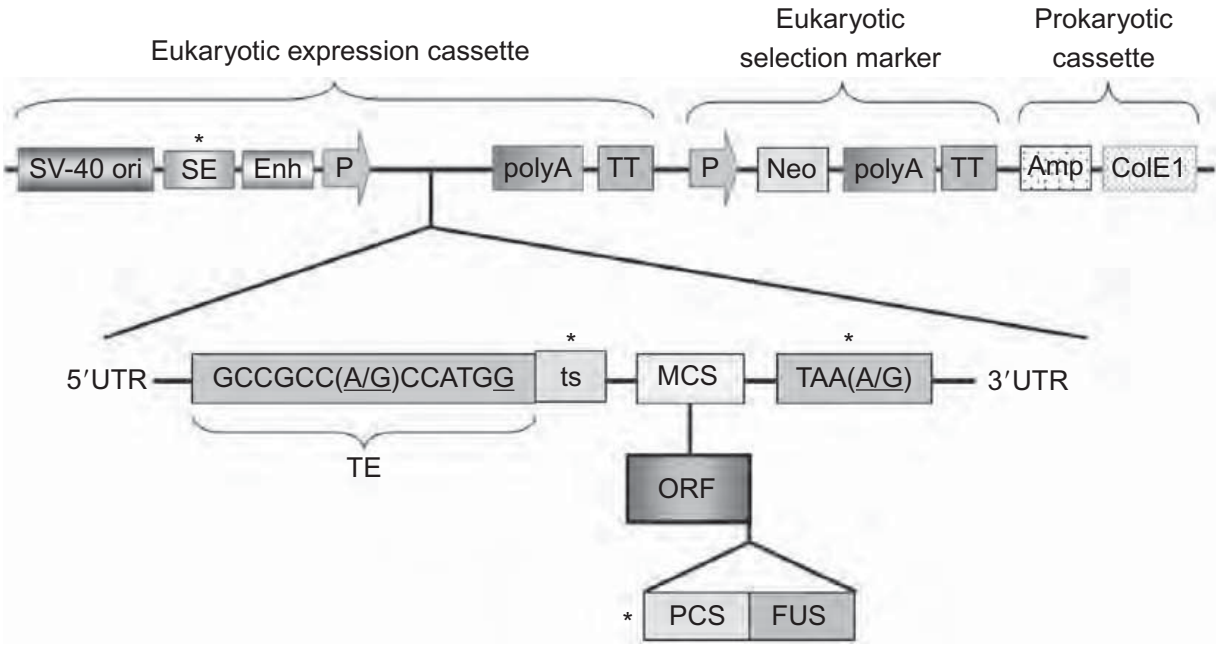


Figure 3.2

Basic and optional components of an expression vector for animal cells. The basic structure of a plasmid vector can be divided into three cassettes: (1) a eukaryotic expression unit containing as basic elements: promoter (p), enhancer (Enh), eukaryotic origin of replication (e.g. SV40 ori) if an episomal replication is preferred, polyadenylation (polyA) and transcription termination signals (TT), and a multiple cloning site (MCS), where the sequence of interest (ORF) is inserted. Other additional elements that can be included are: SARs or LCRs (structural elements, SE), targeting signal (ts), sequence encoding for a fusion protein or tag (FUS) and a protease cleavage site (PCS). The gene to be expressed (ORF) should contain the Kozak sequence or translational enhancer (TE) and an appropriate stop codon (TAA); (2) a biochemical marker (e.g. Neo neomycin phosphotransferase) containing the basic elements that regulate its transcription; and (3) a prokaryotic replication unit, composed of a replication origin (ColE1) and a selection marker (Amp, ampicillin) that allows the genetic manipulation of the vector in *E. coli*. Asterisks indicate optional elements.

stimuli present pleiotropic effects in the host cells, compromising their specificity. To overcome this, regulatory elements of evolutionary distant species are preferred, the most common being the lactose- (Lac), tetracycline- (Tet) or erythromycin- (E; isolated from *E. coli*), streptogramin or pristinamycin-operon/repressor (PIP; originating from *Streptomyces coelicolor* or *S. pristinaespiralis*). Operators are DNA sequences that, bound to specific molecules (polypeptides, metabolites, etc.), can diminish or increase the affinity of the RNA polymerase for the neighboring promoter, thus repressing or promoting the transcriptional activity of the adjacent gene. In the case of the lactose operon, the inducer is isopropyl 1-thio β-D-galactopyranoside (IPTG), which binds to the lactose repressor protein, alters its conformation, and results in the dissociation of the protein from the operon, thus de-repressing the expression of the contiguous gene. One of the disadvantages of this system is that IPTG is toxic at high concentrations (50 mM), restricting its application on a large scale (Makrides, 1999). Ward *et al.* (1995), using a recombinant vaccinia virus, designed a thermoregulable variant of the lac repressor that showed a temperature-dependent

affinity for the operon. The benefit of this system is that the effector of the induction is the culture temperature. A similar inducible system regulated by tetracycline allows the expression of a gene by induction (Tet-On®) or repression (Tet-Off®) in the presence of tetracycline or derivatives. This is accomplished by modifications in the repressor protein, such as mutations and/or fusions to repressors or transactivators. This system has the advantage of low toxicity. Pleiotropic effects are its major drawback, together with the loss of control in the repression (leakiness), evidenced by a basal synthesis of protein in the absence of inducer (Blau and Rossi, 1999).

Other alternatives are based on mutated versions of endogenous elements that have lost the capacity to respond to the endogenous inducers or to bind genomic sequences of animals, for example: (i) the systems modulated by receptors of the steroid hormones that are induced with mifepristone (RU486), an antagonist of progesterone; (ii) artificial chimeras that respond to ecdysone, a hormone that regulates the molting process in insects; or (iii) human immunophilins (FK506 or FRAP), which can be induced by rapamycin (Papadakis *et al.*, 2004).

In some cases, it is preferable that the expression of the target gene occurs in a predetermined phase of the cell cycle or growth and/or in a specific cell type. The recent findings of promoters that fulfill these requirements facilitated synchronizing the expression of recombinant proteins with the cell cycle, and enlarged the list of suitable expression hosts (Blau and Rossi, 1999; Nettelbeck *et al.*, 1999).

Targeting signal sequences and tags. Transport signals guide readily synthesized proteins through the different intracellular trafficking routes and dictate their final location. The targeting signals appear in the form of an N-terminal sequence (15–60 amino acids), which is generally removed when the polypeptide reaches its destiny, and/or as a three-dimensional peptide structure exposed on the surface of the mature protein. The putative locations can be: the cytosol, peroxisomes, nucleus, mitochondria, endoplasmic reticulum, Golgi apparatus, secretory lysosomes, vesicles, or cellular surface. With regard to the production of recombinant proteins in animal cells, it is preferred that the product is secreted to the culture medium, thus simplifying its further isolation and purification. If the gene to be expressed lacks a signal peptide for secretion, this can be either introduced by genetic engineering or incorporated into the expression vector. Some examples of secretion signals are found in the N-terminal sequences of tissue plasminogen activator (tPA) and IgG1. The removal of a targeting sequence from the gene of interest is only advisable: (i) if this signal is not essential for the required post-translational processing of the protein, since these modifications may affect the biological activity of the product; (ii) if the accumulation of the recombinant protein in a given cellular compartment may lead to toxicity or complicate its purification.

Tags comprise sequences that go from a few amino acids (His-, FLAG- or c-myc-tags) to large polypeptides (serum albumin, protein A or G, glutathione S-transferase, growth hormone, GFP, CAT, etc.). The purpose of a tag is to confer a novel property such as an increased expression, solubility, stability, and half-life (e.g. serum albumin, protein G, EBV-1 antigen), a specific affinity to simplify its isolation and purification (e.g.

GST, calmodulin-binding peptide, His-, FLAG-tag, protein A, glycoprotein D of HSV), or to enable its detection and/or selection, i.e. tags based on protein-reporters (β-galactosidase, GFP, CAT, hGH) (Makrides, 1999). Tags can be fused to N- or C-terminal ends of the protein and a site for proteolytic cleavage is commonly included to eliminate the tag upon exploitation of its functionality. The proteases most commonly used are thrombin, enterokinase, factor Xa, and TEV (catalytic domain of Nia, the nuclear inclusion protein from tobacco etch virus).

Positioning effect and site-directed chromosomal integration. The term ''positioning effect'' refers to the factors associated with the structural organization of the chromatin that have an influence on the rate of transcription of a heterologous gene (Zahn-Zabal *et al.*, 2001). These factors involve the condensation state of the chromatin, the direction and location of the foreign gene with respect to other genes, and the structural elements in the chromosomes of the host cell (Wurm, 2004). It must be emphasized that nearly 95% of chromatin appears as heterochromatin, which is transcriptionally inactive. In contrast, the euchromatin is less condensed and transcriptionally active, wherein gene expression is influenced by structural elements (i.e. LCRs, SARs, insulators, boundaries) that determine the transcription of the locus in a cell-specific and/or cell cycle-dependent manner (Bode *et al.*, 2003). Given that chromosomal integration of a heterologous gene is a random process, it becomes easy to understand why the chances for integration into a transcriptionally active region of the genome are low and, hence, why the expression pattern of the recombinant product is heterogeneous and gives rise to clonal variation.

At present, there are three different alternatives to overcome the positioning effect: (i) use of expression vectors containing elements such as insulators, boundaries, SARs, and LCRs, and conserved anti-repressor elements whose function is to generate a transcriptionally favorable environment at the integration site (Zahn-Zabal *et al.*, 2001); (ii) the addition of butyrate or tricostatin to the culture, which block the deacetylation of histones and induce a structural relaxation of the chromatin, making sites that were transcriptionally inactive accessible (Gorman *et al.*, 1983); and (iii) site-directed chromosomal integration of the expression cassette by means of the CRE/LoxP of bacteriophage P1 or FLP/FRT systems (for details see Section 3.6.2).

Expression of multiple genes. In animal cells most of the mRNA is monocistronic and encodes a single protein. In some circumstances, a coordinated expression of two or more heterologous genes may be required. This is the case for the establishment of a stable cell line using a selection marker, for metabolic engineering, or for the expression of protein complexes (i.e. antibodies). Some of the strategies used with that aim include: (i) the expression from a single vector of multiple genes, each one controlled by its own promoter; (ii) the creation of fusion proteins, where the genes are placed in tandem and separated by linkers, the expression being controlled by a single promoter; (iii) the transfection with multiple expression vectors, each one carrying a single and distinct gene and selection marker; (iv) the expression of large monocistronic transcripts containing the different cDNAs of each gene linked by sequences encoding protease cleavage sites, i.e. furine protease, which will subsequently

allow each single polypeptide to be obtained; and (v) the use of IRES (internal ribosomal entry site) elements located upstream of the gene sequence, which allow binding of the ribosomal complex and, therefore, the initiation of the translation of each individual open reading frame in a cap-independent manner (Gurtu *et al.*, 1996).

3.5 Cell lines and biotechnological processes

The choice of a suitable host cell line for recombinant protein production depends on many considerations. On one hand, the host cell must show a high transfectability, be able to transcribe, translate, fold, and process the protein and, if possible, to secrete it to the culture medium. On the other hand, it is advisable that the selected cell line grows in a serum-free medium because recovery of the recombinant protein from culture media with a low protein content is simpler (Mather, 1990). An intrinsic resistance of the cells to shear forces and mechanical damage due to agitation, aeration, and bubbling processes is also desirable. Other aspects to be considered are the post-translational modifications that the protein of interest requires and the optimal culture conditions of the selected cell line (Andersen *et al.*, 2000). In this regard, different studies have demonstrated that changes in the carbohydrate content can alter the antigenicity, stability, solubility, tertiary structure, biological activity, or *in vivo* half-life of the recombinant protein (Delente, 1985). Nowadays, cell lines displaying novel biochemical properties can be generated by means of metabolic engineering. These modifications aim: (i) to allow robust cell growth and/or inhibit apoptosis; (ii) to reduce the secretion of toxic products and/or the consumption of nutrients; (iii) to express glycosyltransferases; and (iv) to control cell proliferation and direct energy metabolism towards protein synthesis (Sanders, 1990). Relevant information about metabolic engineering can be obtained from Kaufmann and Fussenegger (2003).

Although numerous cell lines have been screened for their efficiency as a host system for recombinant protein production, only a few have shown favorable properties for the expression of biopharmaceuticals (Hauser, 1997). Regulatory and economic issues for large-scale production and the intended application of the recombinant protein (diagnosis, therapy, etc.) have to be carefully considered (Makrides and Prentice, 2003). Three mammalian cell lines are now commonly used by the pharmaceutical industry: Chinese hamster ovary (CHO) cells, the murine myeloma SP2/0 and the NS0 cell line (see *Table 3.1*). These cell lines have been used to produce 11 of 21 therapeutic products approved from 1996 to 2000 (Chu and Robinson, 2001).

3.6 Expression in animal cells

Once the DNA encoding the protein of interest is introduced into the cells, expression may be transitory over a period of days or weeks until the DNA is lost from the population. The ability to express the heterologous DNA during a short period of time is called ''transient expression'' (Kauf-

Table 3.1 Cell lines commonly used in the biopharmaceutical industry

Cell line	Source	Applications
COS	Monkey	Transient expression Production of recombinant viruses
HEK 293	Human	Transient and stable expression Production of recombinant viruses
BHK 21	Hamster	Transient and stable expression Vaccine production
CHO.K1, CHO dhfr	Hamster	Transient and stable expression
Hybridomas, NS0, SP2/0	Mouse	Stable expression Monoclonal antibodies production
MDCK	Dog	Stable expression Vaccines production
Per.C6™	Human	Stable expression Production of recombinant viruses and vaccines
Vero	Monkey	Vaccine production
Sf9, Sf21	Insect	Production of recombinant proteins and baculoviruses
Tn 368, High Five® BTI TN 5B1 4	Insect	Recombinant protein production

man, 2000). A few transfected cells may incorporate the exogenous DNA into their genome by a recombination mechanism leading to the ''stable expression'' of a gene (Kaufman, 1997). *Figure 3.3* shows the different phases involving the expression of a heterologous gene.

3.6.1 Transient expression

In general, transient expression allows fast production of a desired protein to characterize it or to verify the integrity, functionality, and efficiency of different recombinant vectors. This represents a convenient means of comparing the performance of different vectors and ensuring their functionality before the more laborious procedure of isolating and characterizing stably transfected cell clones (Kaufman, 1997; Makrides, 2003). Transient expression obviates the clonal variation commonly observed in stable transfections. The production of large amounts of recombinant protein has recently been reported for transient expression systems on large scales (Wurm and Bernard, 1999, 2001; Girard *et al.*, 2002; Derouazi *et al.*, 2004).

Different cell lines can be used for transient expression. These include COS, BHK, CHO, and two variants of genetically modified HEK-293 cells: HEK-293 T (Kim *et al.*, 1997) and HEK-293 EBNA (Cachianes *et al.*, 1993). COS cells were generated by transfection of CV1 cells (African green monkey kidney cells) with a mutant of the SV-40 virus that has a defective replication origin but encodes the T antigen, necessary to initiate viral replication. Hence, only vectors defective in the T antigen and containing an SV-40 replication origin can multiply in these cells (Gluzman, 1981). This host/vector system allows the amplification of the copy number of plasmid DNA (approximately 10 000 copies per cell), yielding

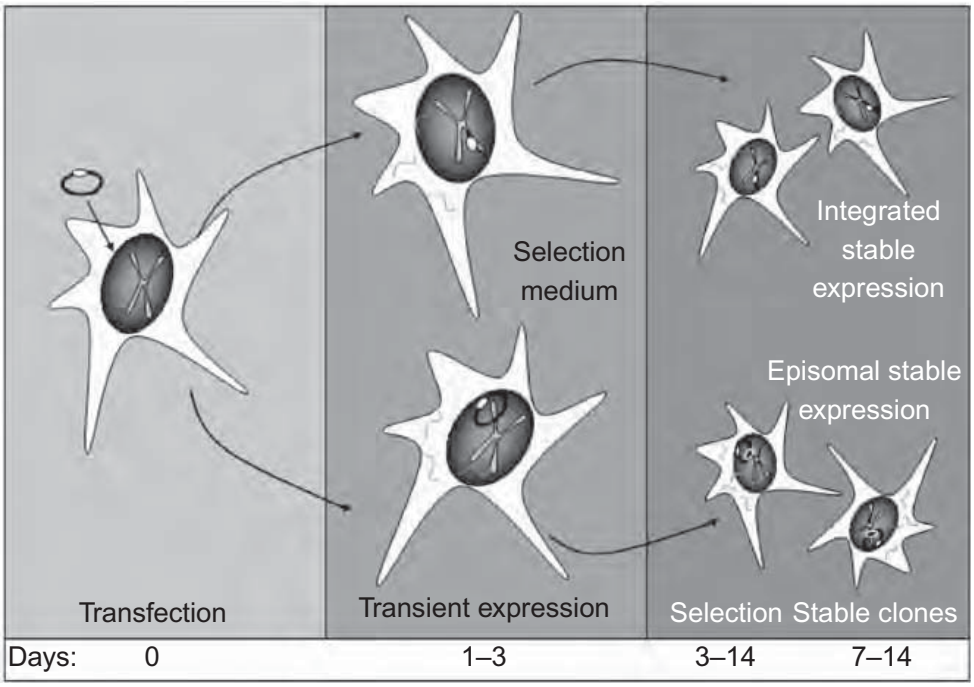


Figure 3.3

Expression of a heterologous gene in mammalian cells. The transfection of host cells with the recombinant vector can be performed by different methods (see Section 3.7). During the initial phase, first to third day upon transfection, the cells express the gene transiently. In the absence of selection agent, the DNA is eventually lost from the cell population. In a small proportion of transfected cells, the exogenous DNA is incorporated, by non homologous recombination, leading to the generation of cell lines that stably express the gene of interest. If the vector contains episomal replication elements, then a stable episomal expression of the exogenous gene takes place. If the vector integrates into the cell genome, then a process known as integrated stable expression occurs.

a high protein expression that is followed by cell lysis a few days after transfection (Kaufman, 1997; Makrides, 2003). A novel cell line (named HBK) showing high transient expression levels was recently generated (Cho *et al.*, 2002). This is a somatic hybrid of two human cell lines, HEK-293 and Burkitt lymphoma cells, which combines high transfectability and capacity for growth in suspension from each cell line, respectively.

3.6.2 Stable expression

In the case of stable transfections, the selection agent is added to the cultures approximately 2 days post-transfection. The selection phase normally lasts days to weeks. Those cells surviving the selection pressure harbor the selection marker, grow as discrete colonies, and can either express or not the protein of interest. These transfectants are soon isolated by means of traditional techniques, which include single cell cloning by cylinders, limiting dilution or soft agar. These methods have nowadays been replaced at the industrial level by fully automated techniques that are faster, less laborious, and enable high-throughput screening, for example,

by flow cytometry and using robots (Wurm, 2004). It is important to stress that generally less than 1% of a cell population that transiently expresses a gene will give rise to stable cell lines (Jordan and Wurm, 2003). *Table 3.2* compares the main advantages and disadvantages of transient and stable expressions.

An alternative and novel strategy for the efficient generation of stable cell lines uses the Cre/LoxP and the FLP/FRT recombination systems to drive a site-specific integration of the heterologous gene. The Cre (cyclization recombination) recombinase from the P1 bacteriophage recombines the DNA in a 34 bp site, known as LoxP (locus of crossover of P1), whereas the FLP recombinase, isolated from the *Saccharomyces cerevisiae* 2 μm plasmid, recognizes the FRT site (FLP recombination target), whose size is 48 bp. The first phase of this method consists of selecting cell lines that have randomly integrated a reporter gene flanked by LoxP or FLP sites into a transcriptionally active chromosomal locus. In a second phase, the reporter gene is replaced by the gene of interest through a recombination process mediated by Cre or FLP recombinases (Makrides, 2003). Recently, Bode *et al.* (2003) have explored the potential of structural elements located in the chromatin to direct a site-specific integration of the gene, and were able to isolate high producer cell lines. A host/vector system that makes use of FLP/FRT elements and different modified cell lines, containing a single FRT site in a transcriptionally active locus, is commercially available (Flp-In™ expression vectors, Invitrogen).

The success of transfection, either stable or transient, depends on several factors that must be taken into account and can be summarized as follows: (i) the transfectability and physiology of the cell line; (ii) the characteristic of the genetic marker in the expression vector; (iii) the type of expression desired; (iv) the size of the expression cassette and the quality of the DNA to be introduced; (v) the compatibility of the transfection method and/or reagents with the cell line; (vi) the type of assay to be used for detection of

Table 3.2 Comparison of transient and stable expression

Transient expression	Stable expression
Short time frame for the generation of the product	Long time frame for the generation of the product
Simple, does not require selection marker	Selection and/or amplification markers are required
No need to screen for recombinant clones	Tedious and time consuming screening of the recombinant clones
Expression levels are not influenced by the ''positioning effect''	The expression levels are influenced by the ''positioning effect'' and the gene copy number
Useful to confirm the integrity of expression vectors, convenient for high throughput screening methods, e.g. to study different mutant genes	Once a stable expressing clone is identified, it constitutes an unlimited source for protein production
Circular DNA is used	The use of linearized or circular DNA depends on the transfection method, but, in general, linearized DNA is advisable

the recombinant product; and (vii) the presence of fetal bovine serum and/or antibiotics in the culture medium. Additionally, the selection of an appropriate vector is as important as the selection of the cell line and/or of the transfection conditions.

3.7 Introduction of DNA into mammalian cells

A wide variety of methodologies and reagents are currently employed to introduce different molecules into eukaryotic cells. The incorporation of DNA can be achieved by two different mechanisms: infection or transfection. The first consists of a biological process mediated by a virus (the viral infection of cells is mediated by receptors), while the second makes use of physical or biochemical methods to incorporate the DNA into the cell. Although the virus-mediated methods are more efficient, they are more laborious and time-consuming compared with transfection. Additionally, the nature of the infection process requires the presence of virus-specific receptors in the host cell to allow viral penetration, which restricts the spectrum of possible host cells. Another limitation of viral infection as a method for DNA transfer is that, unlike plasmid transfection, it is not possible to simultaneously transfer multiple recombinant viruses into the cell (Wurm and Bernard, 1999).

In the next sections, the transfection methods most commonly used in cell culture laboratories will be described. It is difficult to predict the best method for transfection, so the expression vector, the cell type, and the facilities of each laboratory should be taken into account before deciding which technique to apply (Wurm, 2004). For infection techniques, an updated compilation can be obtained from Heiser (2004).

3.7.1 Calcium phosphate co-precipitation method

Although this technique was originally used to increase the infectivity of adenoviral DNA (Graham and Van der Eb, 1973), it became more popular after extending its application to plasmid DNA (Maitland and McDougall, 1977; Wigler *et al.*, 1977). Although the original method has been frequently modified to increase the transfection efficiency, it consists basically of mixing purified DNA with calcium chloride and phosphate buffer at neutral pH, which results in the formation of a fine, visible precipitate. The precipitate (DNA–calcium phosphate complex), when added to the cells, is incorporated by phagocytosis. The complexes fuse to the phagosomes and are transported to different cellular organelles, including the nucleus (Chen and Okayama, 1991). For many cell lines, the transfection efficiency can be further increased by means of short exposures of the cells to chemical agents like glycerol or dimethyl sulfoxide (DMSO) in concentrations between 10 and 20%. Variables such as pH, precipitate quality, incubation time with the precipitate, DNA concentration, cellular state, temperature, and concentrations of chemical agents, can influence the transfection efficiency. This method is widely used because of its low cost, the lack of requirements for sophisticated infrastructure, and its applicability to a wide range of different adherent and suspension cell lines. On

the other hand, cytotoxicity, high mutation rates, and the need for optimization and standardization of the transfection conditions are recognized as the major disadvantages of this method. The critical parameters to obtain an optimal precipitate were very well described by Chen and Okayama (1991) and Jordan *et al.* (1996). Girard *et al.* (2002) reported the establishment of a large-scale transfection method (100 L bioreactor) for HEK-293 cells in suspension using calcium phosphate. More recently, Lindell *et al.* (2004) developed a new technique named calfection, which consists of the addition of a calcium chloride/DNA mixture to the culture medium in agitation. Although this method presents good scale-up potential, the mechanism by which the DNA is incorporated remains unknown, which complicates optimization of the process.

3.7.2 Cationic polymers

Several polycations with a good buffering capacity below physiological pH, such as lipopolyamines and polyamidoamine polymers, have proved to be efficient transfection agents (Boussif *et al.*, 1995). Two of the most popular methods based on polycations are discussed below: diethylaminoethyl-dextran (DEAE-dextran) and polyethylenimine (PEI).

DEAE-dextran. Like the calcium phosphate co-precipitation method, the DEAE-dextran technique was originally developed to increase the viral infectivity of animal cells, and its application was later extended to transfection processes. Although it is simple, efficient, and appropriate for transient expression, its use for stable transfections has not given satisfactory results. The transfection efficiency of this method can be increased by treating cells with glycerol or DMSO. The DNA is incorporated by endocytosis, and thus exposed to extreme pH levels and cellular nucleases, which may explain, to a certain extent, the high frequency of mutations observed when transfecting by this method (Calos *et al.*, 1983). This transfection technique can be applied to both adherent and suspension cell lines. For detailed transfection protocols, the works by Keown *et al.* (1990) and Kaufman (1997, 2000) are recommended.

PEI. Boussif *et al.* (1995) reported for the first time the use of PEI as a vehicle for gene delivery into cells and, since then, there has been a growing interest in the *in vitro* and *in vivo* applications of this method to animal cells. PEI is an organic polymer with a high density of amino groups that can be protonated. At physiological pH, the polycation presents a high affinity for binding DNA and can mediate the transfection of eukaryotic cells (Boussif *et al.*, 1995). PEI presents two types of structures, linear or branched, with variable molecular weights. Linear PEI was reported to display a higher gene transfer efficiency under serum-free conditions in CHO cells in comparison with branched PEI (Derouazi *et al.*, 2004). Moreover, the linear form with a molecular weight of 25 kDa showed the highest level of recombinant protein expression while preventing aggregation and attachment of cells grown in suspension. Although the precise mechanism mediating gene transfer by PEI remains unknown (Bertschinger *et al.*, 2004), it has been postulated that the buffer capacity of the polycation at the lysosomal level would protect the DNA:PEI particles from nuclease degradation (Boussif *et al.*, 1995). The critical

parameters for PEI-mediated transfections are the amount of DNA, the PEI/DNA ratio, the cell density at the time of transfection, and the order of addition of reagents. In fact, Boussif *et al.* (1995) described that the transfection efficiency is one order of magnitude higher when the cationic polymer is added to the plasmid solution rather than vice versa. This method has been demonstrated to be effective for primary cultures and cell lines, for adherent and suspension cells, and for serum-containing and serum-free media. In addition, the cost of the PEI-based method enables its use in transient transfections on a large scale (Boussif *et al.*, 1995; Derouazi *et al.*, 2004).

3.7.3 Lipid-mediated gene transfer (lipofection)

Since the first work of Felgner *et al.* (1987), describing the use of a synthetic cationic lipid for transfection, more than a dozen cationic liposomes have been developed (Gao and Huang, 1995). Under optimal conditions, the cationic lipids form small unilamellar liposomes when formulated in water. The surface of these liposomes is positively charged and interacts with the phosphate backbone of the DNA, forming complexes that present high affinity for the negatively charged surface of the cell membrane. The uptake and delivery of lipid–DNA complexes into the intracellular compartment is mediated by endocytosis. Normally, cationic liposomes contain an amphiphilic cationic lipid (DOSPA, DOTMA, etc.) and a neutral helper lipid, generally DOPE. DOPE is needed in the case of cationic lipids that do not form bilayers in order to stabilize the formation of the cationic liposome (Gao and Huang, 1995). This innovative technology stands out as being easy to accomplish, and its diverse applications offer reproducible results associated with very good yields. The large variety of formulations available has conferred to lipofection a great versatility of applications: stable cell lines and primary cultures, transient and stable transfections, adherent or suspension cells. Different formulations presenting high transfection efficiencies are commercialized by several companies. Unfortunately, the high cost of these products precludes their use in large-scale processes. Multivalent cationic lipids present better transfection efficiencies than those composed of monovalent cationic lipids (Behr *et al.*, 1989). For example, Lipofectamine^{TM} (composed of DOSPA:DOPE, i.e. a multivalent cationic lipid associated with a helper lipid) turned out to be more effective than Lipofectin (DOTMA:DOPE, a monovalent cationic lipid associated with a helper lipid). Both these formulations are commercialized by Invitrogen. Alternatively, the biopharmaceutical company Genentech reported the routine use of a ''home-made'' cationic lipid for transient transfection of CHO cells in a 5–10 L scale (Wurm and Bernard, 2001).

3.7.4 Electroporation

In this method, the delivery of DNA molecules to the cells is mediated by short strong electrical pulses (Wong and Neumann, 1982). The exposure of the cells to an electrical field induces a potential difference across the cellular membranes that generates temporary pores through which the

DNA reaches the cytoplasm. Compared with other methods, such as calcium phosphate co-precipitation and DEAE-dextran, electroporation presents lower mutation frequencies (Bertling *et al.*, 1987), perhaps because the DNA remains free in the cytoplasm and nucleoplasm. Several cell types resistant to transfection by other procedures, including lymphocytes (Potter *et al.*, 1984), hematopoietic stem cells (Toneguzzo and Keating, 1986) and murine embryonic stem cells (Torres and Kühn, 1997), have been successfully electroporated. Furthermore, this technique renders higher efficiencies when linearized DNA is used (Sanders, 1990). The advantages of this technique include its simplicity, reproducibility, applicability for transient and stable transfections, for adherent and suspension cells, and the possibility to control gene copy number in transfectants. On the other hand, the pulse time and the intensity of the electrical field raise concerns and must be determined empirically for each cell type, since succesful transfection can be achieved in a very limited range of voltage. A number of electroporation devices are commercially available, which are safe, easy to handle, and allow the control of different electroporation parameters. In addition to the physical properties of the electrical pulse, the type of buffer solution, and the quality and concentration of cells (exponentially growing cells, between 10^6 and 10^7 cells/ml) have to be considered. In particular, cell concentration has proved to be critical, since lower cell densities reduce the transfection efficiency and higher cell densities favor cell fusion processes that have a detrimental effect on transfection (Spencer, 1991).

3.8 Selection markers

Upon transfection, there are cells that incorporate the plasmid (transfected) and others that do not (wild-type). A selection system allows the separation of these two cell populations. In this regard, the detection of morphologic changes or, more commonly, the use of some toxic metabolite for which the vector confers resistance (biochemical markers) have become the methods of choice to select positive transfectants.

3.8.1 Morphological changes

In general, cells transfected with DNA that contains transforming genes, derived from oncogenic viruses, lose the growth feature called ‘‘contact inhibition,’’ which limits their growth and proliferation. These transfectants can be visualized under the microscope and isolated as colonies.

3.8.2 Biochemical markers and gene amplification

Many genes that encode proteins causing biochemical tranformations in the cells have been isolated, characterized, and employed in eukaryotic expression systems. Among these biochemical markers, those conferring resistance to cytotoxic drugs are among the most commonly used. They allow the selection of stably transfected cells from a heterogeneous population cultured in the presence of toxic metabolites (Sanders, 1990). As

previously described, plasmids employed for stable expression contain a selection cassette with a gene that will provide a new property to the transfected cell. Both the sequence encoding the product and the selection gene can be contained in the same plasmid or in individual vectors, and this has no influence on the expression levels of the recombinant protein. When present in the same vector they can be expressed from a polycistronic mRNA (see Section 3.4.3). One strategy to increase the chances of obtaining high producer cell lines consists of driving the expression of the resistance gene by a weak promoter. Although this approach significantly reduces the efficiency of the transfection, the cells that survive the selective pressure show higher yields of recombinant protein (Wurm, 2004). The resistance to a drug can be recessive or dominant. Those genes providing recessive resistance need a particular host, which must be deficient in the activity by which it will be selected, whereas the genes confering dominant resistance can be used independently of the biochemical background of the cell (Keown *et al.*, 1990). Use of the TK gene is one example of selection employing a recessive biochemical marker. Mammalian cells display an absolute dependency on the TK enzyme when they are cultivated in the presence of aminopterin, which blocks the *de novo* synthesis of thymidine. The selection medium HAT, containing hypoxanthine, aminopterin, and thymidine, is used to rescue cells that express TK. Obviously, this selection system requires cells that lack TK, also identified as TK . In this respect, TK cell lines are available from human and murine origin (Bacchetti and Graham, 1977; Maitland and McDougall, 1977; Wigler *et al.*, 1977).

The selection systems based on a dominant marker are based on resistance to antibiotics. A large number of drugs are used for this purpose: neomycin, hygromycin, puromycin, bleomycin, and zeocin, among others. However, two aminoglycoside phosphotransferases are most routinely used, namely neomycin-phosphotransferase and hygromicin-B-phosphotransferase. Both confer resistance by phosphorylation-mediated inactivation of the corresponding antibiotics. The toxic metabolites added to the culture medium are G418 (analog to gentamicin and also known as geneticin) and hygromycin B, both of which inhibit protein synthesis (González *et al.*, 1978; Colbere-Garapin *et al.*, 1981).

Another group of biochemical markers is characterized by promoting the phenomenon of ''gene amplification'' and, hence, known as amplification markers. Various features have been shown for the gene amplification process: (i) the amplification is obtained after treatment with increasing concentrations of the selection agent; (ii) the amplified DNA shows certain instability; (iii) the size and structure of the amplified unit is variable and temporary (Keown *et al.*, 1990). Within the amplification markers, the most commonly used are the enzymes dihydrofolate reductase (DHFR), involved in the metabolism of nucleotides (*Figure 3.4*), and glutamine synthetase (GS). For DHFR, selection takes place in the absence of hypoxanthine and thymidine, whereas for GS selection takes place in the absence of glutamine. Although most of the ''amplified'' cells express significant amounts of the recombinant protein, the increase in specific productivity (usually 10–20-fold) varies from clone to clone (Wirth *et al.*, 1988). In eukaryotic vectors, the DHFR gene is of prokaryote (*E. coli*) or

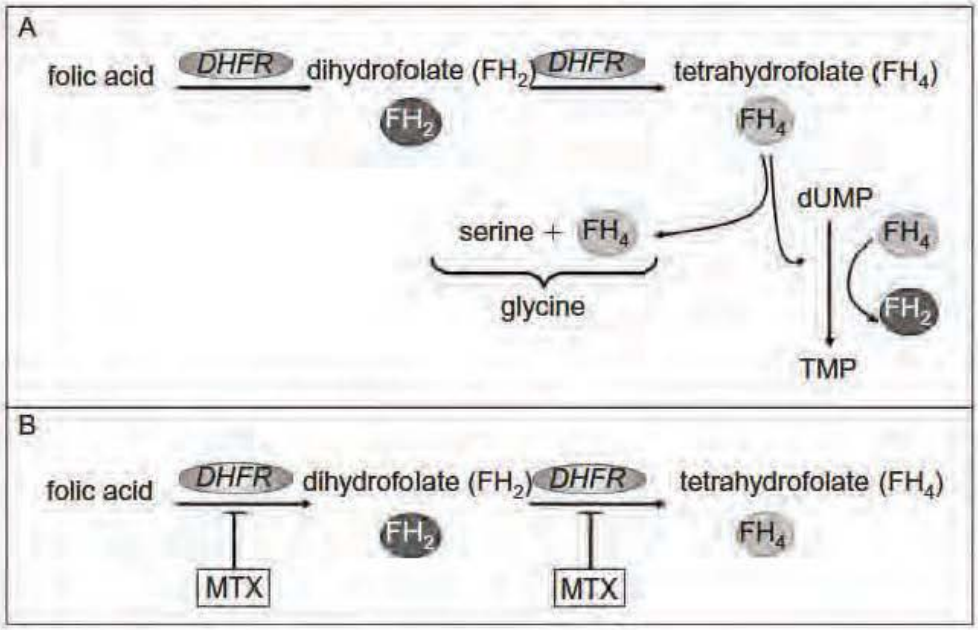


Figure 3.4

Gene amplification. (A) The DHFR enzyme catalyzes the conversion of folate to tetrahydrofolate (FH_4) via the intermediate product dihydrofolate (FH_2). FH_4 is essential for the biosynthesis of purine, glycine, and thymidine monophosphate (TMP) from deoxyuridine monophosphate (dUMP). (B) In the DHFR system, the expression of recombinant protein is increased by exposing the cells to high methotrexate (MTX) concentrations. MTX is an analog of folic acid that binds to DHFR, inhibiting the enzyme and causing cell death. Increasing concentrations of MTX in the culture medium induce a selection pressure for cells with high levels of DHFR and, concomitantly, the gene of interest is amplified. Highly resistant clones commonly contain hundreds to thousands of copies of the plasmid integrated in their chromosomes.

murine origin, the latter producing a protein with higher resistance against MTX (Simonsen and Levinson, 1983). The extensive use of this system lies in the availability of CHO cells lacking the DHFR enzyme (Urlaub and Chasin, 1980). The commonly used clones DUKX-B11 and DG44 can be obtained from Dr Lawrence Chasin (Columbia University, NY).

In the case of the GS, the enzyme catalyzes the synthesis of glutamine from glutamate and ammonium. Ammonia is an undesirable byproduct of cellular metabolism that is released into culture medium. Thus, this system presents a dual effect: on one hand, it reduces the ammonia levels in the culture medium and, on the other hand, it provides cells with a continuous source of glutamine, which is an unstable amino acid. GS is specifically and irreversibly inhibited by methionine sulfoximine (MSX), an analog of glutamine. Therefore, cells overexpressing the enzyme are resistant to high doses of MSX (Sanders and Wilson, 1984). Similarly to the DHFR system, the GS/MSX system has proved to be effective for gene amplification. A typical example of this system is based on NS0 cells transfected with the GS gene and treated with 10–100 μM MSX, which allowed the isolation of resistant clones containing several copies of the GS and the heterologous gene (Bebbington *et al.*, 1992).

Finally, it is worth emphasizing that the optimization of the selection method must precede any attempt to obtain recombinant cell lines. Performing a dose-response assay is highly recommended to assess the lowest concentration of the antibiotic that kills the wild-type cells after a given period of time.

3.8.3 Reporter markers

The advent of reporter markers has greatly contributed to a better understanding of gene expression in eukaryotic cells. A reporter gene encodes for a protein that can be monitored by simple and sensitive methods. Reporter genes are commonly used to: (i) optimize and monitor DNA delivery methods; (ii) study a variety of promoters and regulatory elements, since the expression levels of the reporter protein will reflect the transcription activity; (iii) analyze the traffic and/or the cellular compartmentalization of the studied protein; (iv) characterize transcription factors and associated proteins, such as co-activators; (v) identify protein–protein interactions; and (vi) develop high-throughput screening of compounds with potential therapeutic effects. An ideal reporter gene should meet the following characteristics: (i) to be absent or expressed in very low levels in the cell; (ii) to be detected by a simple, sensitive, and reliable method; (iii) not to induce pleiotropic effects in the host cell. A significant number of reporter genes are suitable for use in mammalian cells, whose expression is evidenced by colorimetric, fluorescence, or chemiluminescence methods. Some examples are described below.

Chloramphenicol acetyl transferase (CAT). This bacterial enzyme was the first reporter protein used for studying the transcriptional activity of eukaryotic regulatory sequences (Gorman *et al.*, 1982). CAT inactivates chloramphenicol, an inhibitor of prokaryotic protein synthesis, by converting it to the mono- or di-acetylated species. Measurement of CAT activity requires a ^{14}C-radiolabeled chloramphenicol or acetyl-CoA and, therefore, an additional step is neccessary to separate the radio-labeled reactant from the product. Novel detection methods based on fluorescent substrates or ELISA assays, which do not use radiolabeled reagents, have been described more recently (Bullock and Gorman, 2000).

Beta-galactosidase (β-gal). The β-gal enzyme from *E. coli* is the most frequently used reporter marker in science, because it is functionally active and well tolerated in a variety of cell lines. β-gal activity can be monitored both qualitatively, by *in situ* staining, and quantitatively, in cell lysates, without the need for sophisticated laboratory equipment. The enzyme hydrolyzes many natural or synthetic β-D-galactopyranosides, which allows the use of different detection methods (Serebriiskii and Golemis, 2000). Because mammalian cells have endogenous β-gal activity, this reporter gene should be used only in cells displaying a low endogenous activity. *In situ* detection of β-gal requires cell fixation and addition of the colorless substrate 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal), which is converted into a blue precipitate by the enzyme action. On the other hand, determination of β-gal activity in cellular extracts is performed by a simple and fast assay based on the conversion of the colorless ortho-nitrophenyl-β-D-galactopyranoside (ONPG) into galactose and ortho-

nitrophenol, which is detected at 420 nm (Miller, 1972). Alternatively, β-gal activity can be assessed *in vivo* using the substrate FdG (fluorescein di-β-D-galactopyranoside). Hydrolysis of this compound releases fluorescein, which is retained intracellularly and can be measured by fluorescent activated cell sorting or FACS (Rossi *et al.*, 2000). Different β-gal detection protocols are reviewed by Alam and Cook (2003).

Luciferase. Luciferase and luciferin are a non-specific enzyme and its substrate, respectively, that upon reaction in the presence of ATP, Mg^{2+}, and O_2 generate bioluminiscence. Different genes or cDNAs encoding luciferases have been isolated from different organisms, such as unicellular seaweed, marine bacteria, and fish. Due to the specificity of the reaction, many luciferases have been used as reporter proteins either in prokaryotic or eukaryotic cells (Greer and Szalay, 2002). Firefly luciferase (LUC) is one of the most widely used. The oxidation reaction produces oxyluciferin, CO_2, and a photon. The intensity of light emitted during the reaction is measured with a luminometer. Luciferase-based techniques are sensitive, simple, rapid, inexpensive, safe (organic solvent or radiolabeled compounds are not required for the test), and applicable to a wide range of cell lines. One of the most recent advances constitutes the development of highly sensitive techniques that make the detection of luciferase activity in living cells and organisms feasible (Greer and Szalay, 2002).

Green fluorescent protein (GFP). GFP was discovered during the investigation of marine bioluminescent organisms. In the 1960s, it was demonstrated that *Aequoria* jellyfish emitted light after the addition of Ca^{2+}, with no need for other cofactors. The gene encoding the *Aequoria* GFP was cloned in 1991, and is used as a reporter in cells, where upon excitation at 395 nm a green fluorescence is emitted at 509 nm. The GFP is exceptionally stable with respect to pH, temperature, and proteolysis, and can be detected in living cells and organisms (Yang *et al.*, 1996). Due to the prolonged time required between the post-translational maturation and the appearence of fluorescence (3–5 hours) in the wild-type GFP (Brand, 1995), different mutants exhibiting an increased fluorescence intensity have been generated (Welsh and Kay, 1997). The enhanced GFP (EGFP) is a variant with two amino acid substitutions (Phe64Leu and Ser65Thr), whose fluorescence is shifted to the red spectrum. The advantages that EGFP presents over the wild-type species are: (i) an increased fluorescence intensity; (ii) a shorter time period between protein synthesis and fluorescence; and (iii) higher expression levels in several animal cells. Currently, there are also yellow (EYFP) and cyan (ECFP) variants, which are excited at 514 and 434 nm, with a maximal emission at 477 and 527 nm, respectively.

Alkaline phosphatase (AP). APs refer to a class of hydrolase enzymes that catalyze the hydrolysis of a broad range of monophosphates and have an optimal activity at alkaline pH (10–10.5). APs are found in all organisms. In earlier times, the use of APs as reporter marker was hampered because most animal cells exhibit high endogenous activity. This limitation was overcome by Berger *et al.* (1988), who generated a secreted form of the human placental AP, known as SEAP. The enzymatic properties of the SEAP are equivalent to the wild-type AP. AP activity derived from SEAP is distinguished from the endogenous AP by assaying the activity in an

aliquot of culture supernatant. In general, SEAP offers certain benefits when compared with other intracellular reporter enzymes: (i) there is no need for cell lysis; (ii) the kinetics of gene expression is easily monitored by harvesting and assaying the supernatant periodically; (iii) living transfected cells remain available for additional phenotypic characterization, such as cell growth, specific mRNA expression, and other enzymatic activities; and (iv) the test presents good sensitivity due to the use of chemiluminiscence substrates (Alam and Cook, 2003)

3.9 Screening, quantitation, and bioassay methods

Once the selection process is completed, many resistant clones may be obtained, which express the heterologous gene at different levels. According to experienced researchers, the expression levels of ''overproducer'' clones should be 5–30-fold higher than the average of the pool of clones. These variations in the expression levels are independent of the cell line and the transfection method used (Wirth and Hauser, 1993). For that reason, it is necessary to have screening and monitoring methods that are sensitive and specific enough to detect and quantify the protein expressed by high producer clones in early stages. In general, protein detection can be carried out by assaying its biological activity (in the case of enzymes, coagulation factors, cytokines, growth factors, hormones, etc.) or by using an immunochemical assay (ELISA, Western blot, RIA, dot blot, etc.). The first provides a precise measurement of the protein functionality, while the second is only useful to identify the molecule without an indication of activity. In general, the immunochemical methods are technically simple, fast, and less costly than those based on the assesment of biological activity.

In the case of recombinant products expressed with a tag or fusion protein, the immunoassay can be directed towards the detection of the tag, for example, in the case of an antibody that reacts against myc-tag (see Section 3.4.3). Another example is the use of bicistronic vectors where, apart from the gene of interest, a reporter gene is coexpressed and its properties are exploited for the selection and isolation of recombinant clones (i.e. GFP-coexpressing cells sorted by flow cytometry). Alternatively, high speed cell sorting can be used for the selection and separation of cells when the reporter gene is expressed as a surface protein, for example, a receptor. In this example, the separation is based on the use of a fluorescent-labeled antibody that reacts against the surface protein.

3.10 Optimizing the initial stage of an animal cell-based bioprocess

In summary, it can be concluded that the optimization of the expression of heterologous genes in animal cells deserves careful design. Firstly, the biological properties of the final product and the amount required for its further application (e.g. diagnostics or therapy) have to be considered. These two factors determine the strategy and expression system that best suit the needs of the recombinant product. Both the vector and the target

sequence can be genetically manipulated, adding or removing elements that can improve the rate of transcription and translation of the gene of interest. The selected host for the production of the recombinant protein must be able to introduce the post-translational modifications required for the biological activity of the product. It is essential that the host cell is cultivated under optimal conditions to warrant higher transfection efficiencies, and to facilitate the selection, cloning and growth of the future recombinant cell lines. Finally, selecting the most suitable transfection method and choosing the selection marker and screening technique are pivotal issues that can substantially increase the probability of isolating recombinant cell lines with high specific productivities.

References

Alam J, Cook JL (2003), Reporter genes for monitoring gene expression in mammalian cells, In: Makrides SC (Ed.), Gene Transfer and Expression in Mammalian Cells, Elsevier Science BV, Amsterdam, pp. 291 308.

Andersen DC, Bridges T, Gawlitzek M, Hoy C (2000), Multiple cell culture factors can affect the glycosylation of Asn 184 in CHO produced tissue type plasmino gen activator, Biotechnol. Bioeng. 70:25 31.

Bacchetti S, Graham FL (1977), Transfer of the gene for thymidine kinase to thymidine kinase deficient human cells by purified herpes simplex virus DNA, Proc. Natl Acad. Sci. U S A 74:1590 1594.

Bebbington CR, Renner G, Thomson S, King D, Abrams D, Yarranton GT (1992), High level expression of a recombinant antibody from myeloma cells using a glutamine synthetase gene as an amplifiable selectable marker, Biotechnology 10:169 175.

Behr JP, Demeneix B, Loeffler JP, Perez Mutul J (1989), Efficient gene transfer in mammalian primary endocrine cells with lipopolyamine coated DNA, Proc. Natl Acad. Sci. U S A 86:6982 6986.

Berger J, Hauber J, Hauber R, Geiger R, Cullen BR (1988), Secreted placental alkaline phosphatase: a powerful new quantitative indicator of gene expression in eukar yotic cells, Gene 66:1 10.

Bertling W, Hunger Bertling K, Cline MJ (1987), Intranuclear uptake and persistence of biologically active DNA after electroporation of mammalian cells, J. Biochem. Biophys. Methods 14:223 232.

Bertschinger M, Chaboche S, Jordan M, Wurm FM (2004), A spectrophotometric assay for the quantification of polyethylenimine in DNA nanoparticles, Anal. Biochem. 334:196 198.

Blasey HD, Lundström K, Tate S, Bernard AR (1997), Recombinant protein produc tion using the Semliki Forest Virus expression system, Cytotechnology 24:65 72.

Blau HM, Rossi FM (1999), Tet B or not tet B: advances in tetracycline inducible gene expression, Proc. Natl Acad. Sci. U S A 96:797 799.

Bode J, Götze S, Ernst E, Hüsemann Y, Baer A, Seibler J, Mielke C (2003), Architecture and utilization of highly expressed genomic sites, In: Makrides SC (Ed.), Gene Transfer and Expression in Mammalian Cells, Elsevier Science BV, Amsterdam, pp. 551 572.

Bourbeau D, Zeng Y, Massie B (2003), Virus based vectors for gene expression in mammalian cells: Adenovirus, In: Makrides SC (Ed.), Gene Transfer and Expres sion in Mammalian Cells, Elsevier Science BV, Amsterdam, pp. 109 124.

Boussif O, Lezoualc′h F, Zanta MA, Mergny MD, Scherman D, Demeneix B, Behr JP (1995), A versatile vector for gene and oligonucleotide transfer into cells in culture and in vivo: polyethylenimine, Proc. Natl Acad. Sci. U S A 92:7297 7301.

Brand A (1995), GFP in *Drosophila*, Trends Genet. 11:324 325.
Bullock C, Gorman C (2000), Fusions to chloramphenicol acetyltransferase as a reporter, Methods Enzymol. 326:202 221.
Burton EA, Bai Q, Goins WF, Fink D, Glorioso JC (2003), Virus based vectors for gene expression in mammalian cells: Herpes simplex virus, In: Makrides SC (Ed.), Gene Transfer and Expression in Mammalian Cells, Elsevier Science BV, Amster dam, pp. 27 54.
Cachianes G, Ho C, Weber RF, Williams SR, Goeddel DV, Leung DW (1993), Epstein Barr virus derived vectors for transient and stable expression of recombi nant proteins, Biotechniques 15:255 259.
Calos MP, Lebkowski JS, Botchan MR (1983), High mutation frequency in DNA transfected into mammalian cells, Proc. Natl Acad. Sci. U S A 80:3015 3019.
Carroll MW, Kovacs GR (2003), Virus based vectors for gene expression in mamma lian cells: Vaccinia virus, In: Makrides SC (Ed.), Gene Transfer and Expression in Mammalian Cells, Elsevier Science BV, Amsterdam, pp. 125 136.
Chen C, Okayama H (1991), Calcium phosphate mediated gene transfer into estab lished cell lines, In: Murray EJ (Ed.), Gene Transfer and Expression Protocols, Humana Press, Clifton, pp. 15 21.
Cho MS, Yee H, Chan S (2002), Establishment of a human somatic hybrid cell line for recombinant protein production, J. Biomed. Sci. 9:631 638.
Chu L, Robinson D (2001), Industrial choices for protein production by large scale cell culture, Curr. Opin. Biotechnol. 12:180 187.
Colbere Garapin F, Horodniceanu F, Kourilsky P, Garapin A (1981), A new dominant hybrid selective marker for higher eucaryotic cells, J. Mol. Biol. 150:1 14.
Condreay JP, Kost TA (2003), Virus based vectors for gene expression in mammalian cells: Baculovirus, In: Makrides SC (Ed.), Gene Transfer and Expression in Mammalian Cells, Elsevier Science BV, Amsterdam, pp. 137 150.
Condreay JP, Witherspoon SM, Clay WC, Kost TA (1999), Transient and stable gene expression in mammalian cells transduced with a recombinant baculovirus vector, Proc. Natl Acad. Sci. U S A 96:127 132.
Cone RD, Mulligan RC (1984), High efficiency gene transfer into mammalian cells: generation of helper free recombinant retrovirus with broad mammalian host range, Proc. Natl Acad. Sci. U S A 81:6349 6353.
Delente JJ (1985), Glycosylation revisited, Trends Biotechnol. 3:218.
Derouazi M, Girard P, Van Tilborgh F, Iglesias K, Muller N, Bertschinger M, Wurm FM (2004), Serum free large scale transient transfection of CHO cells, Biotech nol. Bioeng. 87:537 545.
Emerman M, Temin HM (1984), Genes with promoters in retrovirus vectors can be independently suppressed by an epigenetic mechanism, Cell 39:449 467.
Enjuanes L, Almazán F, Ortego J (2003), Virus based vectors for gene expression in mammalian cells: Coronavirus, In: Makrides SC (Ed.), Gene Transfer and Expres sion in Mammalian Cells, Elsevier Science BV, Amsterdam, pp. 151 168.
Fedorov A, Saxonov S, Gilbert W (2002), Regularities of context dependent codon bias in eukaryotic genes, Nucleic Acids Res. 30:1192 1197.
Felgner PL, Gadek TR, Holm M, Roman R, Chan HW, Wenz M, Northrop JP, Ringold GM, Danielsen M (1987), Lipofection: a highly efficient, lipid mediated DNA transfection procedure, Proc. Natl Acad. Sci. U S A 84:7413 7417.
Fromm M, Berg P (1983), Simian virus 40 early and late region promoter functions are enhanced by the 72 base pair repeat inserted at distant locations and inverted orientations, Mol. Cell. Biol. 6:991 999.
Gao X, Huang L (1995), Cationic liposome mediated gene transfer, Gene Therapy 2:710 722.
Gerasimova TI, Corces VG (2001), Chromatin insulators and boundaries: effects on transcription and nuclear organization, Annu. Rev. Genet. 35:193 208.

Girard P, Derouazi M, Baumgartner G, Bourgeois M, Jordan M, Jacko B, Wurm FM (2002), 100 liter transient transfection, Cytotechnology 38:15 21.
Gluzman Y (1981), SV40 transformed simian cells support the replication of early SV40 mutants, Cell 23:175 182.
Goff SP, Berg P (1976), Construction of hybrid viruses containing SV40 and lambda phage DNA segments and their propagation in cultured monkey cells, Cell 9:695 705.
González A, Jimenez A, Vazquez D, Davies JE, Schindler D (1978), Studies on the mode of action of hygromycin B, an inhibitor of translocation in eukaryotes, Biochem. Biophys. Acta 521:459 469.
Gorman CM, Moffat LF, Howard BH (1982), Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells, Mol. Cell. Biol. 2: 1044 1051.
Gorman CM, Howard BH; Reeves R (1983), Expression of recombinant plasmids in mammalian cells is enhanced by sodium butyrate, Nucleic Acids Res. 11: 7631 7648.
Graham FL, Van Der Eb AJ (1973), A new technique for the assay of infectivity of human adenovirus 5 DNA, Virology 52:456 467.
Gray NK, Wickens M (1998), Control of translation initiation in animals, Annu. Rev. Cell. Dev. Biol. 14:399 458.
Greer LF, Szalay AA (2002), Imaging of light emission from the expression of luciferases in living cells and organisms: a review, Luminiscence 17:43 74.
Grens A, Scheffler IE (1990), The 5′ and 3′ untranslated regions of ornithine decarboxylase mRNA affect the translational efficiency, J. Biol. Chem. 265:11810 11816.
Gurtu V, Yan G, Zhang G (1996), IRES bicistronic expression vectors for efficient creation of stable mammalian cell lines, Biochem. Biophys. Res. Commun. 229:295 298.
Hamer DH, Davoli D, Thomas CA Jr, Fareed GC (1977), Simian virus 40 carrying an *Escherichia coli* suppressor gene, J. Mol. Biol. 112:155 182.
Hauser H (1997), Heterologous expression of genes in mammalian cells, In: Hauser H, Wagner R (Eds), Mammalian Cell Biotechnology in Protein Production, Walter de Gruyter, Berlin, pp. 3 32.
Heiser WC (2004), Gene delivery to mammalian cells, vol. 2: Viral gene transfer techniques, In: Heiser W (Ed.), Methods in Molecular Biology, vol. 246, Humana Press, Totowa.
Holmes Son ML, Appa RS, Chow SA (2001), Molecular genetics and target site specificity of retroviral integration. Adv. Genet. 43:33 69.
Huang MT, Gorman CM (1990), The simian virus 40 small t intron, present in many common expression vectors, leads to aberrant splicing, Mol. Cell. Biol. 10: 1805 1810.
Inácio A, Liebhaber SA (2003), Pathways of mammalian messenger RNA degradation, In: Makrides SC (Ed.), Gene Transfer and Expression in Mammalian Cells, Elsevier Science BV, Amsterdam, pp. 495 512.
Izant JG, Weintraub H (1985), Constitutive and conditional suppression of exogenous and endogenous genes by anti sense RNA, Science 229:345 352.
Jordan M, Schallhorn A, Wurm FM (1996), Transfecting mammalian cells: optimiza tion of critical parameters affecting calcium phosphate precipitate formation, Nucleic Acids Res. 24:596 601.
Jordan M, Wurm FM (2003), Co transfer of multiple plasmid/viruses as an attractive method to introduce several genes in mammalian cells, In: Makrides SC (Ed.), Gene Transfer and Expression in Mammalian Cells, Elsevier Science BV, Amster dam, pp. 337 348.
Kaufman RJ (1990), Vectors used for expression in mammalian cells, Methods

Enzymol. 185:487 511.
Kaufman RJ (1997), DNA transfection to study translational control in mammalian cells, Methods 11:361 370.
Kaufman RJ (2000), Overview of vector design for mammalian gene expression, Mol. Biotechnol. 16:151 160.
Kaufmann H, Fussenegger M (2003), Metabolic engineering of mammalian cells for higher protein yield, In: Makrides SC (Ed.), Gene Transfer and Expression in Mammalian Cells, Elsevier Science BV, Amsterdam, pp. 457 469.
Keown WA, Campbell CR, Kucherlapati RS (1990), Methods for introducing DNA into mammalian cells, Methods Enzymol. 185:527 537.
Kim CH, Oh Y, Lee TH (1997), Codon optimization for high level expression of human erythropoietin (EPO) in mammalian cells human or yeast codon usage effect on over expression in 293T cell culture, Gene 199:293 301.
King LA, Possee RD (1992), The Baculovirus Expression System: A Laboratory Guide, In: King LA, Possee RD (Eds), Chapman & Hall, New York.
Klehr D, Maass K, Bode J (1991), Scaffold attached regions from the human interferon beta domain can be used to enhance the stable expression of genes under the control of various promoters, Biochemistry 30:1264 1270.
Kozak M (2005), Regulation of translation via mRNA structure in prokaryotes and eukaryotes, Gene 361:13 37.
Levinson AD (1990), Expression of heterologous genes in mammalian cells, Methods Enzymol. 185:485 487.
Lindell J, Girard P, Muller N, Jordan M, Wurm FM (2004), Calfection: a novel gene transfer method for suspension cells, Biochim. Biophys. Acta 1676:155 161.
Luckow VA (1995), Protein production and processing from baculovirus expression vectors, In: Shuler ML, Wood HA, Granados RR, Hammer DA (Eds), Baculo virus Expression Systems and Biopesticides, J Wiley & Sons, New York, pp. 51 90.
Lundstrom K (2003), Virus based vectors for gene expression in mammalian cells: Semliki Forest virus, In: Makrides SC (Ed.), Gene Transfer and Expression in Mammalian Cells, Elsevier Science BV, Amsterdam, pp. 207 230.
Lutfalla G, Armbruster L, Dequin S, Bertolotti R (1989), Construction of an EBNA producing line of well differentiated human hepatoma cells and of appropriate Epstein Barr virus based shuttle vectors, Gene 76:27 39.
McCaughan KK, Brown CM, Dalphin ME, Berry MJ, Tate WP (1995), Translational termination efficiency in mammals is influenced by the base following the stop codon, Proc. Natl Acad. Sci. U S A 92:5431 5435.
Mackett M, Smith GL, Moss B (1982), Vaccinia virus: a selectable eukaryotic cloning and expression vector, Proc. Natl Acad. Sci. U S A 79:7415 7419.
Maitland NJ, McDougall JK (1977), Biochemical transformation of mouse cells by fragments of herpes simplex virus DNA, Cell 11:233 241.
Makrides SC (1999), Components of vectors for gene transfer and expression in mammalian cells, Protein Expr. Purif. 17:183 202.
Makrides SC (2003), Vectors for gene expression in mammalian cells, In: Makrides SC (Ed.), Gene Transfer and Expression in Mammalian Cells, Elsevier Science BV, Amsterdam, pp. 9 26.
Makrides SC, Prentice HL (2003), Why choose mammalian cells for protein produc tion?, In: Makrides SC (Ed.), Gene Transfer and Expression in Mammalian Cells, Elsevier Science BV, Amsterdam, pp. 1 8.
Mather JP (1990), Optimizing cell and culture environment for production of recombi nant proteins, Methods Enzymol. 185:567 577.
Matsushita T, Elliger S, Elliger C, Podsakoff G, Villarreal L, Kurtzman GJ, Iwaki Y, Colosi P (1998), Adeno associated virus vectors can be efficiently produced without helper virus, Gene Ther. 5: 938 45.

Mattaj IW (1990), Splicing stories and poly(A) tales: an update on RNA processing and transport, Curr. Opin. Cell. Biol. 3:528 538.
Merrihew RV, Clay WC, Condreay JP, Witherspoon SM, Dallas WS, Kost TA (2001), Chromosomal integration of transduced recombinant baculovirus DNA in mam malian cells, J. Virol. 75:903 909.
Miller JH (1972), Assay of β galactosidase, In: Miller JH (Ed.), Experiments in Molecular Genetics, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, pp. 352 355.
Moss B (1996), Poxviridae: the viruses and their replication, In: Fields BN, Knipe DM, Howley PM (Eds), Fields Virology, 3rd ed., Lippincott Raven, Philadelphia, pp. 2637 2671.
Muller WJ, Mueller CR, Mes AM, Hassell JA (1983), Polyomavirus origin for DNA replication comprises multiple genetic elements, J. Virol. 47:586 599.
Munroe D, Jacobson A (1990), Tales of poly(A): a review, Gene 91:151 158.
Nettelbeck DM, Jerome V, Muller R (1999), A dual specificity promoter system combining cell cycle regulated and tissue specific transcriptional control, Gene Ther. 6:1276 1281.
Niwa H, Yamamura K, Miyazaki J (1991), Efficient selection for high expression transfectants with a novel eukaryotic vector, Gene 108:193 199.
Novina CD, Roy AL (1996), Core promoters and transcriptional control, Trends Genet. 12:351 355.
Papadakis ED, Nicklin SA, Baker AH, White SJ (2004), Promoters and control elements: designing expression cassettes for gene therapy, Curr. Gene Ther. 4: 89 113.
Parolín C, Palú G (2003), Virus based vectors for gene expression in mammalian cells: Retrovirus, In: Makrides SC (Ed.), Gene Transfer and Expression in Mammalian Cells, Elsevier Science BV, Amsterdam, pp. 231 250.
Petitclerc D, Attal J, Theron MC, Bearzotti M, Bolifraud P, Kann G, Stinnakre MG, Pointu H, Puissant C, Houdebine LM (1995), The effect of various introns and transcription terminators on the efficiency of expression vectors in various cultured cell lines and in the mammary gland of transgenic mice, J. Biotechnol. 40:169 178.
Potter H, Weir L, Leder P (1984), Enhancer dependent expression of human kappa immunoglobulin genes introduced into mouse pre B lymphocytes by electropora tion, Proc. Natl Acad. Sci. U S A 81:7161 7165.
Rossi FM, Blakely BT, Blau HM (2000), Interaction blues: protein interactions monitored in live mammalian cells by beta galactosidase complementation, Trends Cell. Biol. 10:119 122.
Sambrook J, Fritsch EF, Maniatis T (2000), Molecular Cloning: A Laboratory Manual, 3rd ed., Cold Spring Harbor Laboratory Press, Nova York, 3 vols.
Sanders PG (1990), Protein production by genetically engineered mammalian cell lines, In: Spier RE, Griffiths JB (Eds), Animal Cell Biotechnology, vol. 4, Academic Press, London, pp. 15 70.
Sanders PG, Wilson RH (1984), Amplification and cloning of the Chinese hamster glutamine synthetase gene, EMBO J. 3:65 71.
Sandig V, Lieber A, Strauss M (1997), Vectors for gene transfer and expression in animals cells, In: Hauser H, Wagner R (Eds), Mammalian Cell Biotechnology in Protein Production, Walter de Gruyter, Berlin, pp. 65 85.
Serebriiskii IG, Golemis EA (2000), Uses of lacZ to study gene function: evaluation of beta galactosidase assays employed in the yeast two hybrid system, Anal. Bio chem. 285:1 15.
Shimotohno K, Temin HM (1981), Formation of infectious progeny virus after insertion of herpes simplex thymidine kinase gene into DNA of an avian retro virus, Cell 26:67 77.

Simonsen CC, Levinson AD (1983), Isolation and expression of an altered dihydrofo late reductase cDNA, Proc. Natl Acad. Sci. U S A 80:2495 2499.
Spencer SC (1991), Electroporation technique of DNA transfection, In: Murray EJ (Ed.), Methods in Molecular Biology, vol. 7, Humana Press, Clifton, pp. 45 52.
Strauss JH, Strauss EG (1994), The alphaviruses: gene expression, replication, and evolution, Microbiol. Rev. 58:491 562.
Sugden B, Marsh K, Yates J (1985), A vector that replicates as a plasmid and can be efficiently selected in B lymphoblasts transformed by Epstein Barr virus, Mol. Cell. Biol. 5:410 413.
Szutorisz H, Dillon N, Tora L (2005), The role of enhancers as centres for general transcription factor recruitment, Trends Biochem. Sci. 30:593 599.
Teshigawara K, Katsura Y (1992), A simple and efficient mammalian gene expression system using an EBV based vector transfected by electroporation in G2/M phase, Nucleic Acids Res. 20:2607 2611.
Toneguzzo F, Keating A (1986), Stable expression of selectable genes introduced into human hematopoietic stem cells by electric field mediated DNA transfer, Proc. Natl Acad. Sci. U S A 83:3496 3499.
Torres RM, Kühn R (1997), Laboratory protocols for conditional gene targeting, In: Torres RM, Kühn R (Eds), Oxford University Press, Oxford, pp. 1 167.
Urlaub G, Chasin LA (1980), Isolation of Chinese hamster cell mutants deficient in dihydrofolate reductase activity, Proc. Natl Acad. Sci. U S A 77:4216 4220.
Ward GA, Stover CK, Moss B, Fuerst TR (1995), Stringent chemical and thermal regulation of recombinant gene expression by vaccinia virus vectors in mammalian cells, Proc. Natl Acad. Sci. U S A 92:6773 6777.
Welsh S, Kay SA (1997), Reporter gene expression for monitoring gene transfer, Curr. Opin. Biotechnol. 8:617 622.
West AG, Gaszner M, Felsenfeld G (2002), Insulators: many functions, many mechan isms, Genes Dev. 16:271 288.
Wigler M, Silverstein S, Lee LS, Pellicer A, Cheng VC, Axel R (1977), Transfer of purified herpes virus thymidine kinase gene to cultured mouse cells, Cell 11: 223 232.
Wirth M, Hauser H (1993), Genetic engineering of animal cells, In: Rehm HJ, Reed G, Pühler A, Stadler P (Eds), Biotechnology, vol. 2, Editora VCH, Weinheim, pp. 663 744.
Wirth M, Bode J, Zettlmeissl G, Hauser H (1988), Isolation of overproducing recombinant mammalian cells by a fast and simple selection procedure, Gene 73:419 426.
Wong TK, Neumann E (1982), Electric field mediated gene transfer, Biochem. Biophys. Res. Commun. 107:584 587.
Wurm FM (2004), Production of recombinant protein therapeutics in cultivated mammalian cells, Nat. Biotechnol. 22:1393 1398.
Wurm FM, Bernard A (1999), Large scale transient expression in mammalian cells for recombinant protein production, Curr. Opin. Biotech. 10:156 159.
Wurm FM, Bernard A (2001), Transient gene expression from mammalian cells: a new chapter in animal cell technology, Cytotechnology 35:155 156.
Xiao X (2003), Virus based vectors for gene expression in mammalian cells: adeno associated virus, In: Makrides SC (Ed.), Gene Transfer and Expression in Mam malian Cells, Elsevier Science BV, Amsterdam, pp. 93 108.
Yaniv M (1982), Enhancing elements for activation of eukaryotic promoters, Nature 297:17 18.
Yang F, Moss LG, Phillips GN Jr (1996), The molecular structure of green fluorescent protein, Nat. Biotechnol. 14:1246 1251.
Zahn Zabal M, Kobr M, Girod PA, Imhof M, Chatellard P, de Jesus M, Wurm FM, Mermod N (2001), Development of stable cell lines for production or regulated

expression using matrix attachment regions, J. Biotechnol. 87:29 42.
Zhu ZY, Veldman GM, Cowie A, Carr A, Schaffhausen B, Kamen R (1984), Construc tion and functional characterization of polyomavirus genomes that separately encode the three early proteins, J. Virol. 51:170 180.

Cell metabolism and its control in culture

4

Paola Amable and Michael Butler

4.1 Introduction

Metabolism, the sum of all chemical transformations taking place in a cell, is a highly coordinated network of activities in which many multi-enzyme systems and pathways are involved. The purposes of this network can be summarized as follows:

(i) to obtain chemical energy by capturing solar energy or degrading energy-rich nutrients from the environment;
(ii) to convert nutrient precursor molecules into the characteristic macromolecules of the cell;
(iii) to polymerize monomers into macromolecules (proteins, nucleic acids, and polysaccharides);
(iv) to synthesize and degrade biomolecules required for specialized cellular functions, such as membrane lipids, intracellular messengers, and pigments.

The network operates through a series of enzyme-catalyzed reactions that constitute the metabolism. Each of the consecutive steps in a metabolic pathway brings about a specific chemical change, usually the removal, transfer, or addition of a particular atom or functional group. The precursor is converted into a product through a series of metabolic intermediates called metabolites. The term intermediary metabolism is often applied to the combined activities of all the metabolic pathways that interconvert precursors, metabolites, and products of low molecular weight.

Some metabolic pathways are linear, some are cyclic, and some are branched, yielding multiple end products from a single precursor or converting several precursors into a single product.

Metabolic pathways are regulated at several levels within a cell. The most immediate regulation is by the availability of substrate. A second type of rapid control is allosteric regulation by a metabolic intermediate or coenzyme (an amino acid or ATP, for example) that signals the cell's internal metabolic state. In multicellular organisms the metabolic activities of different tissues are also regulated and integrated by growth factors and hormones that act from outside the cell (Nelson and Cox, 2004).

Cells, removed from animal tissues or whole animals, will continue to grow if supplied with nutrients and growth factors, allowing single cells to act as independent units that are able to divide by mitosis. Cell lines can

live *in vitro* for a finite time (finite cell lines) or indefinitely (permanent cell lines). Permanent cell lines may continue growth in a culture vessel until limited by some parameter associated with the mode of culture. The limitation for growth in a culture may arise from nutrient depletion, accumulation of metabolic byproducts or lack of surface for anchorage-dependent cells.

Multiple culture passages can be established by transfer of cells from a high density growth-limited culture into fresh medium. In this way, growth of cells can be maintained for prolonged periods in culture. Normal animal cells have an intrinsic finite growth capacity but some cells acquire a capacity for infinite growth and such a population can be called an established or continuous cell line. This requires the cell to go through a process called transformation, which causes the cells to lose their sensitivity to the stimuli associated with growth control. Transformed cells may lose their anchorage dependence and often show some chromosome fragmentation. This genetic state is referred to as aneuploidy, which means that there is slight alteration from the normal diploid state. The transformed cells have a high capacity for growth in relatively simple growth media and without the need for growth factors (Butler, 2004).

4.2 Energy sources

In contrast to plant cells, which normally get their cellular energy from photosynthesis, animal cells need a carbohydrate source, usually glucose, and the amino acid glutamine. The catabolism of these substrates allows the production of two coenzymes (ATP and NADH – nicotinamide adenine dinucleotide), which are essential for maintaining the viability of the cells. These coenzymes can be used for the maintenance, metabolism and/or for the synthesis of particular desired products (Wagner, 1997).

The nature and concentration of carbon and nitrogen substrates in the culture medium affect both the energy status and metabolism of the cells. Medium generally contains both a carbohydrate (normally glucose) and a range of amino acids to satisfy the cellular growth requirements (Butler, 2004). Lipids are also important, not only as an energy source, but also as precursors for the synthesis of the cellular membrane components.

As a consequence of transformation, cells in culture assume a modified metabolism when compared with the same cell in the organism of origin. For example, the consumption rates of glucose and glutamine are very high initially but decline throughout a normal batch culture, as does the specific growth rate. These high uptake rates exceed the cellular need for both precursors and energy, and result in increased fluxes in glycolysis and glutaminolysis, which in turn leads to overflow metabolism and formation of byproducts such as lactate, ammonium, and alanine (Doverskog *et al*., 1997).

4.2.1 Glucose

Glucose is a monosaccharide sugar that functions as an important fuel for energy metabolism in all mammalian cells. It is also a remarkably versatile

precursor, capable of supplying a huge array of metabolic intermediates for biosynthetic reactions. In animals and vascular plants, glucose may be stored (as a polysaccharide such as glycogen or starch), oxidized to a three-carbon compound (pyruvate) via glycolysis to provide ATP and metabolic intermediates, or oxidized via the pentose phosphate cycle (PPC) to yield ribose 5-phosphate for nucleic acid synthesis and reduced nicotinamide adenine dinucleotide phosphate (NADPH) generation for reductive biosynthetic processes.

The cytoplasmic membrane of mammalian cells is impermeable to glucose, and therefore the uptake is by means of transport proteins located in the plasma membrane. In the majority of mammalian cells the glucose transport mechanism is facilitated diffusion. This is saturable, bidirectional, and driven by the concentration gradient across the cell membrane. Once glucose enters the cell cytoplasm, it is rapidly phosphorylated by hexokinase to glucose-6-phosphate. This enzymatic reaction leaves low amounts of free glucose in the cytoplasm, stimulating the transport of more glucose in the uptake direction. Six different isoforms of glucose transporters have been isolated in different tissues and designated GLUT1, GLUT2, GLUT3, GLUT4, GLUT5, and GLUT7 (Craik and Elliot, 1979; Kayano *et al*., 1988; Carruthers, 1990; Gould *et al*., 1991; Burant *et al*., 1992; Kahn, 1992; Pessin and Bell, 1992; Waddell *et al*., 1992).

In the cytosol, glucose-6-phosphate is converted by two main pathways: glycolysis and the PPC.

Glycolysis occurs completely in the cytosol and does not require O_2. It produces a small amount of ATP, NADH, and the three-carbon compound pyruvate. The sequence of reactions is shown in *Figure 4.1*

Four molecules of ATP are formed per glucose molecule during glycolysis, catalyzed by phosphoglycerate kinase and pyruvate kinase. However, early in the glycolytic pathway, two ATP molecules are consumed: one by the addition of a phosphate residue to glucose in the reaction catalyzed by hexokinase and another by the addition of a second phosphate to fructose 6-phosphate in the reaction catalyzed by phosphofructokinase-1. Thus glycolysis yields a net of only two ATP molecules per glucose molecule.

The overall chemical equation for this first stage of glucose metabolism is:

$$C_6H_{12}O_6 + 2NAD^+ + 2ADP^3 + 2P^2 \rightarrow 2C_3H_4O_3 + 2NADH + 2ATP^4$$

The PPC allows the generation of NADPH reduction equivalents required for cell anabolism, and ribose 5-phosphate molecules for the synthesis of nucleic acids. Alternatively, ribose 5-phosphate can also be generated or transformed into fructose 6-phosphate or glyceraldehyde 3-phosphate, providing metabolic flexibility to the cell, in order to balance the fluxes through these pathways. The flux through the PPC is related to the nucleic acid requirements for DNA duplication or RNA transcription, and could probably be controlled by the cell cycle (Wagner, 1997).

In the presence of oxygen, however, pyruvate formed in glycolysis is transported into mitochondria, where it is oxidized by O_2 to CO_2 and H_2O in a series of oxidation reactions, collectively called the tricarboxylic

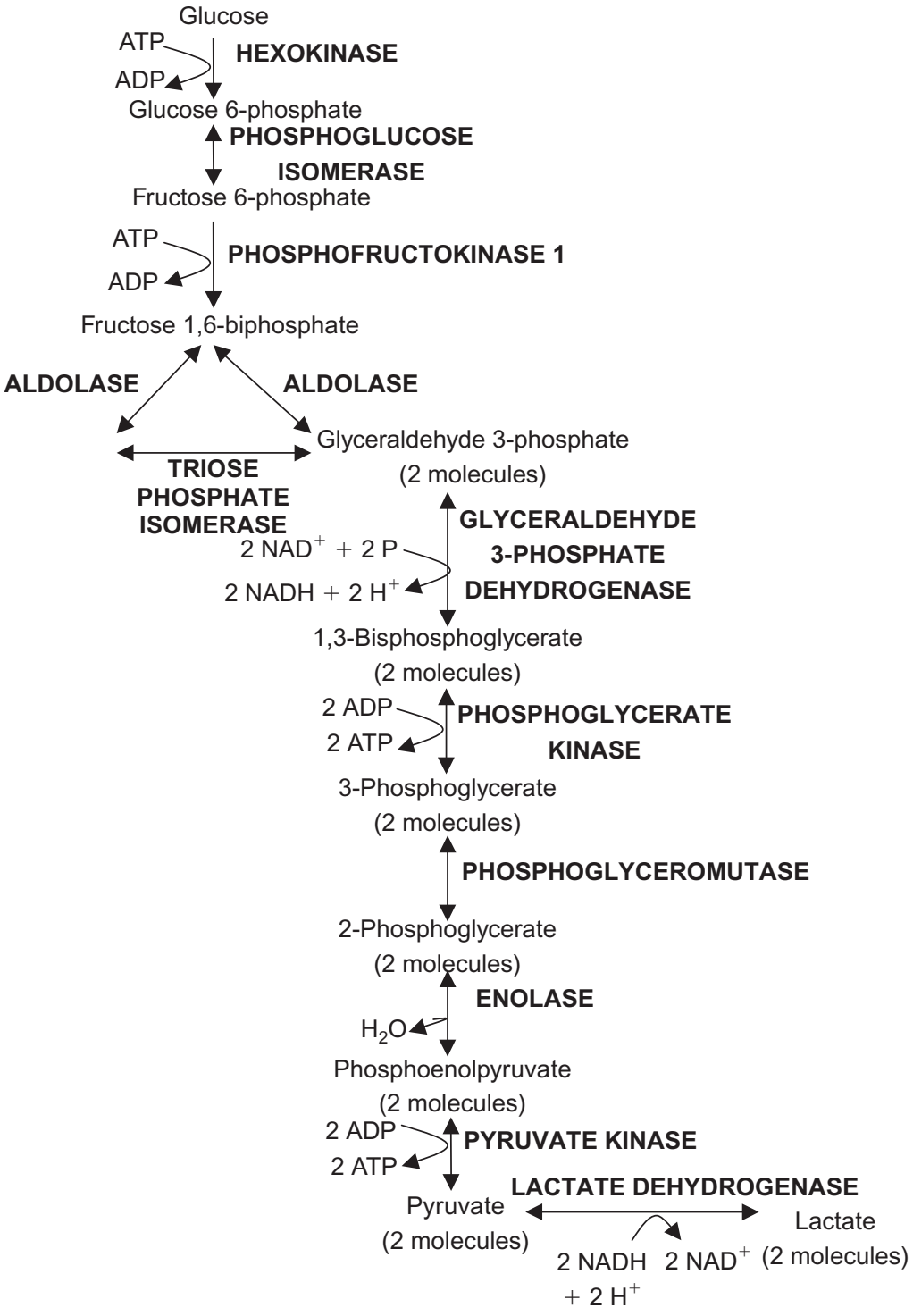


Figure 4.1

The glycolytic pathway by which glucose is degraded to pyruvate.

acid (TCA) cycle, citric acid cycle, or Kreb's cycle (*Figure 4.2*). These reactions generate 8 NADH, 2 GTP (guanosine 5′-triphosphate; interchangeable with ATP), and 2 $FADH_2$ (1,5-dihydro-flavin adenine dinucleotide). When NADH and $FADH_2$ are further metabolized through

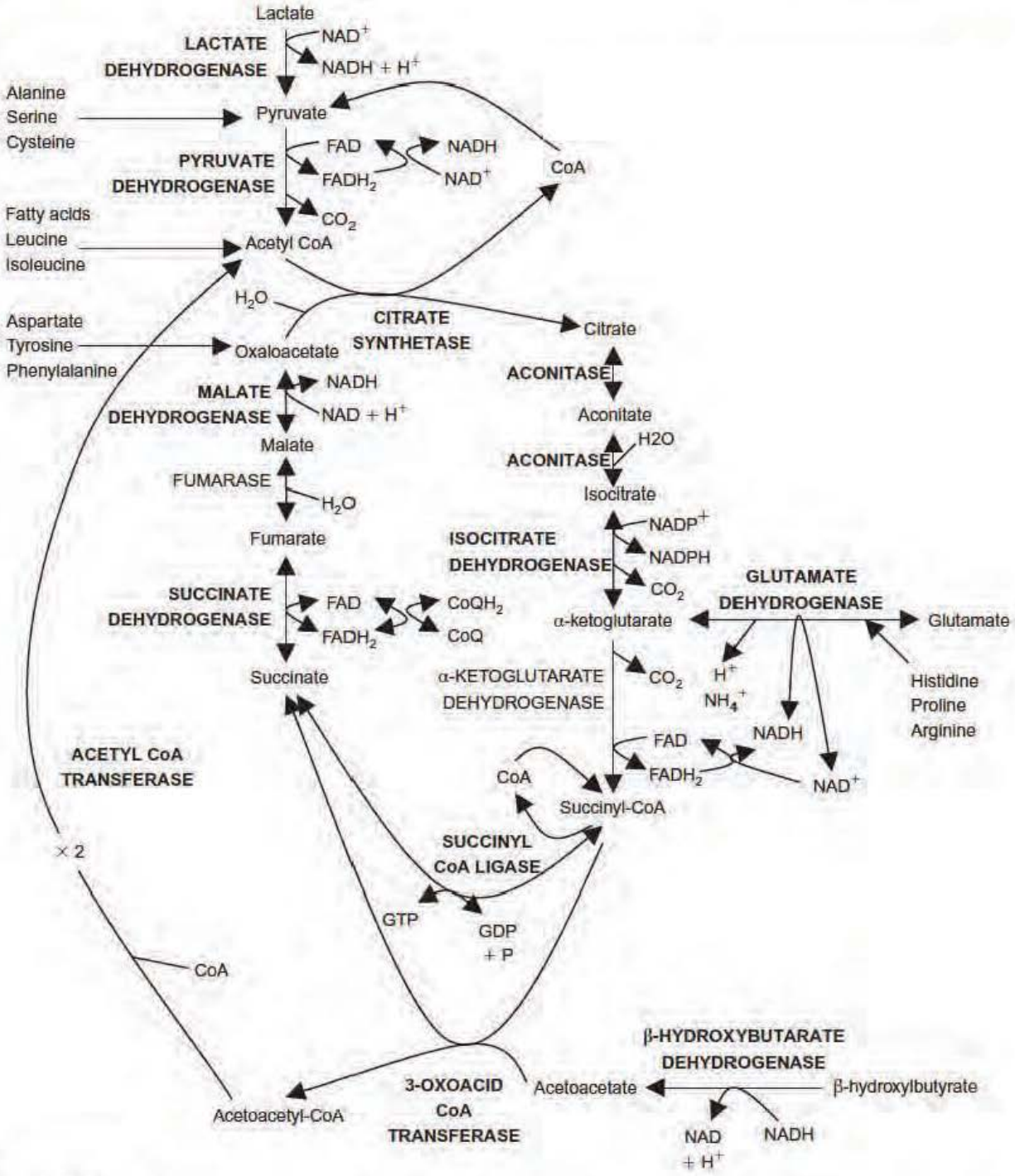


Figure 4.2

The tricarboxylic acid (TCA) cycle, in which acetyl groups transferred from acetyl CoA are oxidized to carbon dioxide.

the oxidative phosphorylation and GTP is converted into ATP, the TCA cycle renders a final amount of 25 ATP molecules per glucose molecule, far outstripping the ATP yield from glycolysis. ATP is the most important molecule for capturing and transferring free energy in biological systems.

The released energy of almost all the metabolic pathways is converted to the chemical energy of phosphoanhydride bonds in ATP. Cells use the energy released during hydrolysis of the ATP bonds to power energetically unfavorable processes.

The TCA cycle takes place inside the mitochondria. It is not only the metabolic pathway that accounts for the complete combustion of the product of glycolysis, but also the pathway that accounts for the complete combustion of carbohydrates, fatty acids, and amino acids. The main functions of the cycle are to provide different compounds that are precursors for the cell anabolism and to generate most of the metabolic energy.

The connection between TCA and glycolysis is made through pyruvate. The pyruvate produced in glycolysis is transported from the cytosol across the mitochondrial membranes to the inner mitochondrial matrix. There, it reacts with coenzyme A (CoA), forming CO_2 and the intermediate acetyl-CoA. This reaction, catalyzed by pyruvate dehydrogenase, is highly exergonic and essentially irreversible.

Although the TCA cycle directly generates only one ATP per turn, there are four oxidation steps in the cycle that provide NADH and $FADH_2$. These compounds generate a large flow of electrons into the respiratory chain and thus lead to formation of a large number of ATP molecules during oxidative phosphorylation. In this pathway, passage of two electrons from NADH or $FADH_2$ to O_2 drives the formation of ATP. The number of ATP molecules that may be formed through oxidative phosphorylation may vary depending on the cell line. It is considered that the metabolism of a NADH molecule generates 2.5 ATP molecules, and a $FADH_2$ molecule generates 1.5 ATP molecules (Elliott and Elliott, 2001). These values are known as P/O ratios and represent the relationship between ATP synthesis and oxygen consumption. Then, the overall yield of ATP from the complete oxidation of glucose is 32 ATP molecules per glucose molecule when both pyruvate molecules are completely oxidized.

Unlike mammalian cells *in vivo*, established mammalian cell lines are highly deregulated. They show a high glycolytic flux (Donelly and Scheffler, 1976) and are unable to completely oxidize glucose, generating high amounts of lactate (a maximum of 2 moles of lactate per mole of glucose) as an end product, even under fully aerobic conditions. Furthermore, in the presence of non-limiting glucose concentrations they consume glucose at a much higher rate than would be strictly required to maintain cell growth. This indicates that a low percentage of pyruvate is incorporated into the TCA cycle for a complete oxidation, a pathway much more efficient regarding the energetic yield (32 ATP instead of 2 ATP when lactate is the end product).

There are several hypotheses related to lactate generation in mammalian cells in culture (Häggström, 2000):

(i) the cells convert pyruvate to lactate as a way to re-equilibrate their redox potential and as a means of NADH regeneration in the cytosol;
(ii) lactate dehydrogenase competes with pyruvate dehydrogenase for conversion of pyruvate to lactate in the cytosol;
(iii) too low concentrations of aspartate in the cytosol may limit the malate–aspartate shuttle activity;

(iv) respiration may be suppressed by low availability of ADP (adenosine diphosphate), which would be consumed due to rapid flux of the glycolysis pathway;
(v) malate may accumulate inside the mitochondria due to glutamine metabolism, which would make more difficult the influx of malate into the mitochondria, as required by the malate–aspartate shuttle;
(vi) the respiratory system may be saturated by NADH generated by the metabolism of glutamine inside the mitochondria;
(vii) there may be a deficiency in a key enzymatic activity linking the glycolysis pathway with the TCA cycle, such as pyruvate dehydrogenase complex, phosphoenolpyruvate carboxykinase, or pyruvate carboxylase. In this situation, the metabolism of pyruvate through the lactate dehydrogenase enzyme would be favored (Fitzpatrick *et al*., 1993; Petch and Butler 1994; Neerman and Wagner, 1996).

From all the hypotheses mentioned above, the low flux of pyruvate into the TCA cycle is better explained by a decreased activity of several enzymes: pyruvate carboxylase, pyruvate phosphoenol carboxykinase, pyruvate dehydrogenase, and malic enzyme II (*Figure 4.3*). Then, the pyruvate accumulated is converted to lactate by the enhanced catalytic action of the lactate dehydrogenase, as an alternative pathway to generate energy for cellular processes.

There are many studies supporting this hypothesis. Gstraunthaler *et al*. (1999) have demonstrated that when renal proximal tubular cells are brought into culture, they revert from oxidative metabolism and gluconeogenesis to high rates of glycolysis. They investigated the metabolism of continuous renal cell lines LLC-PK(1) (porcine kidney) and OK (opossum kidney) and found that glucose was quantitatively converted to lactate, which accumulated in a 2:1 molar ratio (lactate:glucose). In LLC-PK(1) cells, increased glucose supply caused increases in hexokinase, phosphofructokinase, pyruvate kinase, and lactate dehydrogenase activities. This higher lactate dehydrogenase activity is responsible for the increased lactate generation and the reduced TCA cycle activity. Neerman and Wagner (1996) compared glucose metabolism in a mammalian (BHK) and an insect (Sf9) cell line. They found no activity of the key enzymes connecting glycolysis with the TCA cycle, such as pyruvate dehydrogenase, pyruvate carboxylase, and phosphoenolpyruvate carboxykinase, indicating that glucose is mainly metabolized via glycolysis and lactate formation.

Glutamine is normally another important energy source in cultured cell lines. However, in the absence of glutamine, the cells may change their metabolism, to increase the energy yield from glucose. Chen and Harcum (2005) demonstrated that, when Chinook salmon embryo (CHSE) cells were cultured in a glutamine-free culture, hexokinase activity remained constant and lactate dehydrogenase activity decreased. In this way, the lack of glutamine reduced lactate production to make the energy metabolism more efficient.

Acetyl-CoA enters the TCA cycle by reacting with oxaloacetate to form citrate. At this point, there is an important flux of citrate leaving the TCA cycle and being exported to the cytosol, where it contributes to lipid

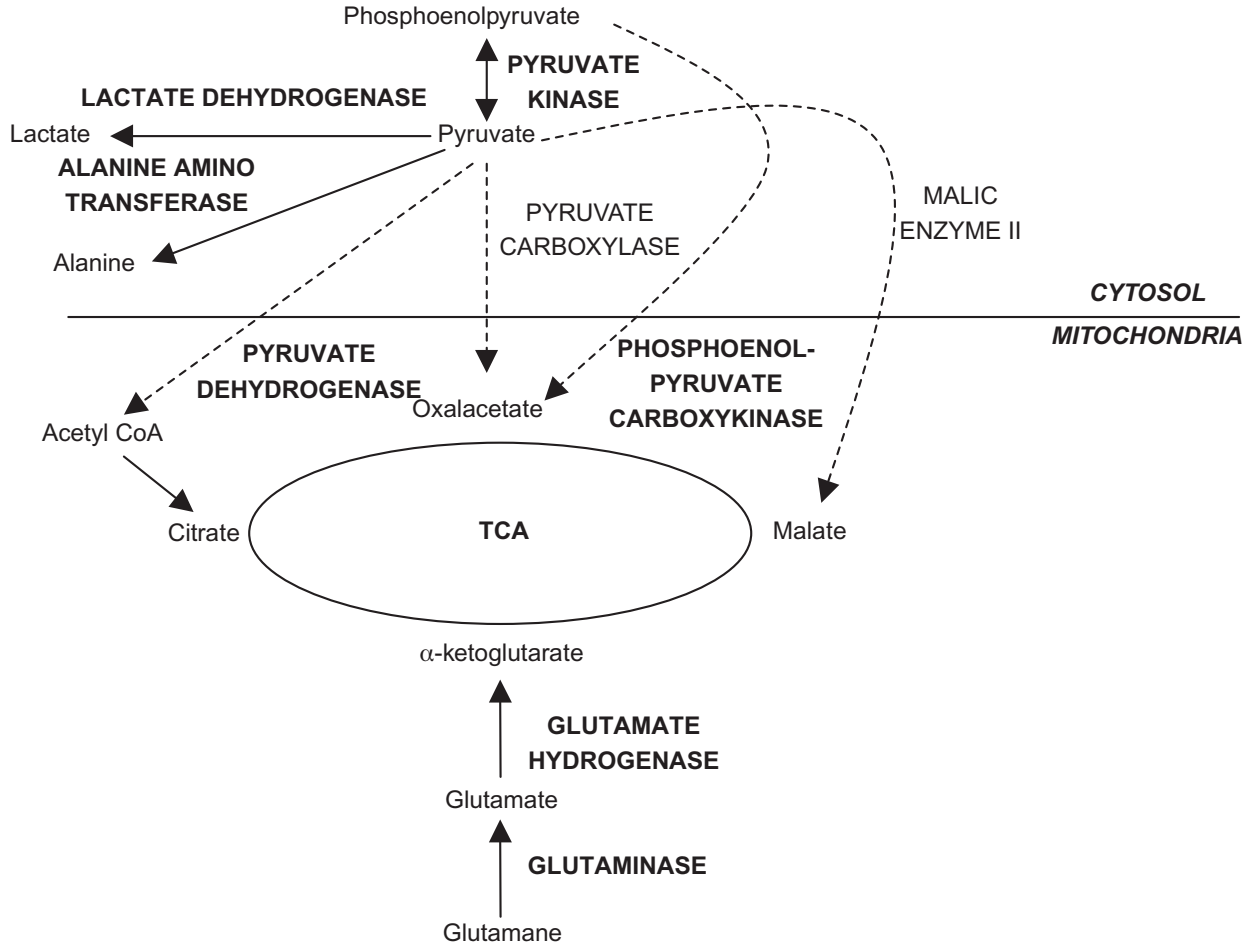


Figure 4.3

Enzymes that link glycolysis and the tricarboxylic acid (TCA) cycle. Dashed arrows indicate reduced or suppressed enzymatic activity.

formation. The combination of a low incorporation of acetyl-CoA into the cycle and the outflow of citrate depletes the metabolites of the TCA cycle, which need to be replenished to satisfy requirements for energy generation, since the ATP production by glycolysis is low. Replenishment may occur at the level of α-ketoglutarate. The primary role of glutamine is to supply this intermediate to keep the operation of the cycle. In this way, glutamine is a major source of carbon and energy. Other amino acids and lipids also participate in the supply of intermediates to the TCA cycle through anaplerotic reactions (*Figure 4.2*).

In most media formulations for mammalian cell lines, glucose is included at an initial concentration of 10–25 mM, which decreases to around half the concentration level within the period of a batch culture. The production of lactic acid may lower the pH of the culture and this may decrease the cellular growth rate. However, if the culture is appropriately buffered then the accumulated lactate levels typically found in a batch culture should not cause any growth inhibition (Hassell *et al*., 1991).

As glucose is metabolized at a far greater rate than it is needed to maintain viability, it is better to supplement it during the culture than to

increase the initial concentration. This will limit the accumulation of lactate in the culture. Nevertheless, the cell concentration and the protein productivity may be affected by glucose concentration. Meijer and van Dijken (1995) have reported the effects of the glucose supply on growth and metabolism of an SP2/0 derived recombinant myeloma cell line in a chemostat culture with Iscove's Modified Dulbecco's Medium (IMDM) medium. Lowering glucose concentration of the feed medium from 25.0 to 1.4 mM resulted in a decrease of steady-state viable cell concentration, whereas viability remained above 90%. Sun and Zhang (2001a) have reported the effects of the glucose concentration on batch cultures of recombinant CHO (Chinese hamster ovary) cells. They demonstrated that the accumulated concentration of erythropoietin (EPO) increased with the increase of glucose concentration from 8.9 to 17.9 mM, and decreased with its further increase from 17.9 to 49.6 mM. They concluded that there is an optimal glucose concentration for the enhancement of EPO expression by these recombinant CHO cells. They also demonstrated that the yield coefficient of lactate from glucose increased with the initial glucose concentrations from 8.9 up to 17.9 mM, and then kept constant above 17.9 mM.

An alternative is to substitute glucose by other hexoses, like galactose or fructose, or by disaccharides, like maltose. Galactose is converted into galactose-6-phosphate, a reaction catalyzed by hexokinase, and then it is transformed into glucose-6-phosphate, an intermediate of glycolysis. Fructose can also be phosphorylated to fructose-6-phosphate by hexokinase. Fructose can also enter the glycolysis pathway at the level of glyceraldehyde-3-phosphate, via the fructose-1-phosphate pathway. As maltose is a disaccharide of glucose, it is slowly hydrolyzed into glucose molecules and then transported into the cell and metabolized. The metabolism of alternative carbohydrate sources provides different amounts of energy. The flux through glycolysis may be slower due to a low affinity of the hexokinase enzyme for galactose and fructose or due to the slow hydrolysis of maltose. Thus, substitution of glucose with fructose, galactose, or maltose may decrease the rate of production of lactic acid (Butler and Christie, 2004).

The reduced glycolytic rate and formation of lactic acid may result in a slower growth rate (Griffiths, 2000). Duval *et al*. (1992) cultured a mouse hybridoma cell line (VO 208) in batch/fed-batch cultures in a medium supplemented with fructose instead of glucose and demonstrated an increase of the lifespan of the culture and an enhancement in the antibody secretion. However, it has been reported that complete glucose substitution by other hexoses can alter product glycosylation (Paredes *et al*., 1999).

Another strategy to improve the efficiency of central carbon metabolism and to reduce lactate accumulation is metabolic engineering. Increasing the flux of glucose into the TCA cycle can improve the efficiency of glucose for energy metabolism by enhanced formation of ATP. Weidemann *et al*. (1994) have reported that the introduction of a cytosolic pyruvate carboxylase derived from *Saccharomyces cerevisiae* into BHK-21 cells enabled cells to transfer glycolysis-derived pyruvate into malate, which then entered the TCA cycle for complete oxidation. As a result, higher yields of recombinant EPO were achieved by the BHK cells.

4.2.2 Glutamine

Glutamine is the major source of energy, carbon, and nitrogen for mammalian cells. It plays two important functions: it acts as an energy donor and plays a critical role in nitrogen metabolism, acting as a collection point for amino groups. For example, in the cytosol of hepatocytes, amino groups from most amino acids are transferred to α-ketoglutarate to form glutamate, which enters the mitochondria and gives up its amino group to form ammonium. Excess ammonium generated in most tissues other than the liver is converted to the amide nitrogen of glutamine. Glutamine and glutamate, or both, are present in higher concentrations than other amino acids in most tissues or biological fluids.

Glutamine is transported into the cytosol of the cell by means of two kinds of transporters: Na^+-dependent and Na^+-independent. Once in the cytosol, glutamine must be transported into the mitochondria, where it is metabolized.

The metabolic pathway of glutamine, called glutaminolysis, begins with an enzymatic reaction catalyzed by glutaminase, which converts glutamine into glutamate with ammonium removal. Then, glutamate is converted into α-ketoglutarate by the action of the glutamate dehydrogenase, liberating another ammonium ion (*Figure 4.4*). This α-ketoglutarate enters directly to the TCA cycle (*Figure 4.2*).

In culture media for mammalian cells, together with the carbohydrate source, glutamine is the most abundant source of reduced carbon. Similar to glucose, glutamine is also consumed by mammalian cells at a high rate, particularly if the glutamine concentration is higher than the minimal needs for maintaining cell viability. This leads to the unwanted accumulation of ammonium ions, a toxic byproduct, in addition to an inefficient use of glutamine.

Glutamine is normally included at a concentration of 1–5 mM, which is a significantly higher concentration than that of any other amino acids. Glutamine is an important precursor for the synthesis of purines, pyrimidines, amino sugars, and asparagine. However, glutamine also has an important role as substrate for the TCA cycle (Butler, 2004).

For some mammalian cells, glutamine is the main source of energy. It was observed in an antibody-secreting murine hybridoma (CC9C10) culture that, after 2 days of exponential growth in batch culture in a medium containing 20 mM glucose and 2 mM glutamine, the glutamine content of the medium was completely depleted, whereas the glucose content was reduced to only 60% of the original concentration. Petch and Butler (1994) demonstrated that glucose is normally metabolized via

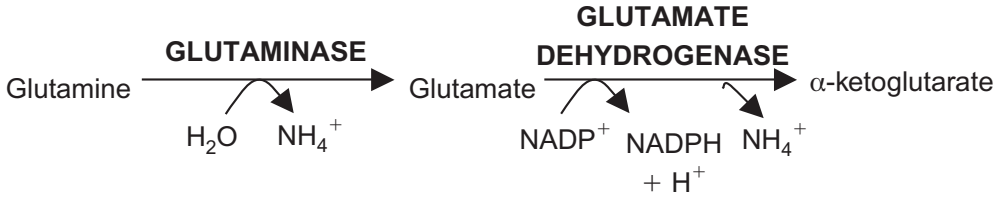


Figure 4.4

Initiation of the glutamine metabolism pathway.

glycolysis (> 96%), the PPC (3.6%), and the TCA cycle (0.6%). Glutamine, on the other hand, is partially oxidized via glutaminolysis to alanine (55%), aspartate (3%), glutamate (4%), lactate (9%), and CO_2 (22%) (Petch and Butler, 1994). Fitzpatrick *et al*. (1993) demonstrated that, in a murine B-lymphocyte hybridoma (PQXB1/2) batch culture, glutamine was completely depleted, but glucose was partially depleted to only 50% of its original concentration when the cells reached a stationary phase following exponential growth. This suggests that glutamine is the major contributor of cellular energy. Vriezen *et al*. (1997) cultured two different cell lines (MN12, a mouse–mouse hybridoma, and SP2/0-Ag14, a mouse myeloma) in steady-state chemostat cultures fed with IMDM medium with 5% serum and glutamine concentrations ranging from 0.5 to 4 mM. The culture profiles showed that glutamine was the growth-limiting substrate in this concentration range. Furthermore, they showed that excess glutamine gives rise to high consumption rates: in glutamine-limited cultures, the specific rates of ammonia and alanine production were low compared with glutamine-rich cultures containing 4 mM glutamine in the feed medium.

The preferential utilization of glutamine as an energy source is cell-dependent. Maranga and Goochee (2006) have demonstrated that PER.C6™ cells fall into a minor category of mammalian cell lines for which glutamine plays a minor role in energy metabolism.

Although glutamine is used as a major substrate for the growth of mammalian cells in culture, it suffers from some disadvantages. The metabolic deamination of glutamine leads to ammonia, which accumulates in the culture up to 2–4 mM and can be inhibitory to cell growth. The problem of ammonia accumulation is made worse by the fact that glutamine may decompose spontaneously to produce ammonia in the culture medium at a rate of 0.1 mM per day at 37°C. The extent of growth inhibition by ammonia is greater at higher pH levels and is cell line-dependent.

The accumulation of ammonia from glutamine can be decreased by a continuous feed of a low concentration of glutamine into the culture. Ljunggren and Häggström (1992) cultured the murine myeloma cell line Sp2/0-Ag 14 in an ordinary batch culture and in a glutamine-limited fed-batch culture. In batch culture, the overflow metabolism of glutamine led to excess production of ammonium and the amino acids alanine, proline, ornithine, asparagine, glutamate, serine, and glycine. In the fed-batch culture, glutamine limitation halved the cellular ammonium production and reduced the ratio of ammonium/glutamine. The excess production of alanine, proline, and ornithine was reduced by a factor of 2–6, while asparagine was not produced at all. They demonstrated that essential amino acids were used more efficiently in the fed-batch culture as judged by the increase in the cellular yield coefficients in the range of 1.3–2.6 times for 7 of the 11 amino acids consumed. Altogether, this leads to a more efficient use of the energy sources glucose and glutamine, as revealed by an increase in the cellular yield coefficient for glucose by 70% and for glutamine by 61%, as well as a reduction in the generation of ammonium.

Maranga and Goochee (2006) have reported that, when PER.C6™ cells were cultured in suspension in a 2-liter serum-free bioreactor at a con-

trolled glutamine concentration of 0.25 mM, the consumption rate of glutamine and the production rate of ammonium were reduced by approximately 30%, with respect to the same culture with a higher glutamine concentration. Furthermore, the consumption rate of alanine and the consumption rate of non-essential amino acids were reduced by 85% and 50%, respectively. The fed-batch control of glutamine also reduced the overall accumulation of ammonium ion by approximately 50% by minimizing the spontaneous thermal degradation of glutamine.

In order to reduce the ammonium production, substitution of glutamine by an alternative substrate such as glutamate may be possible. However, if glutamate is used, a period of adaptation is required during which the activity of glutamine synthetase and the rate of transport of glutamate both increase, since glutamate has a low efficiency transport system. The cell yield increases when ammonia accumulation is decreased following culture supplementation with glutamate rather than glutamine. However, some cell lines fail to adapt to growth in glutamate (McDermott and Butler, 1993). The cell line HL-60 was adapted to growth in glutamine-deficient medium by stepwise glutamine deprivation. The successful adaptation of different cell lines to non-ammoniagenic medium has been described by Hassell and Butler (1990). They replaced glutamine by either glutamate or α-ketoglutarate. A mole to mole substitution of glutamine by glutamate was successful for a McCoy cell line and led to normal growth rates after approximately 10 days. Cell yield was increased by 17%, ammonia accumulation was reduced by 70%, and glucose consumption and lactate production both decreased by more than 70%. A BHK and a Vero cell line had to be slowly adapted from an initially high glutamate concentration. The MDCK cell line could not be adapted to growth on glutamate. The authors proposed that the glutamate uptake, and not the glutamine synthetase activity, is responsible for the ability of a given cell line to grow in a glutamine-free medium (McDermott and Butler, 1993).

An alternative to cell adaptation is metabolic engineering. Cells transformed with the glutamine synthetase gene can grow in media supplemented with glutamate instead of glutamine, with a direct elimination/reduction of ammonia generation, either by glutamine decomposition or metabolism (Paredes *et al*., 1999). Bell *et al.* (1992, 1995) successfully obtained a murine hybridoma cell line that could grow in the complete absence of glutamine by transformation with the glutamine synthetase gene. Ammonia concentrations in the medium of batch cultures of these cells were below detection levels. This cell line could not be adapted to glutamine-free growth even in the presence of elevated levels of glutamate, so metabolic engineering was the unique alternative.

Another strategy for the reduction of ammonia accumulation can be the use of glutamine-containing dipeptides, which hydrolyze slowly in the culture (Butler and Christie, 2004). The supplementation of a glutamine-free medium with dipeptides containing glutamine allows a reduction in the rate of ammonium generation, but this requires the presence of dipeptidases, which may be produced by the cells and may be released into the medium. Christie and Butler (1994) grew a murine hybridoma (CC9C10) in media containing the dipeptides alanyl-glutamine (Ala-Gln) or glycyl-glutamine (Gly-Gln) as a substitute for glutamine. They

obtained high cell yields in the presence of 6 mM Ala-Gln or 20 mM Gly-Gln, with the final cell yield in Gly-Gln 14% higher than in Gln. The higher concentration of Gly-Gln was necessary for cell growth because of the presence of a peptidase (in the cytosolic fraction of the cells) with a lower affinity for Gly-Gln. Monoclonal antibody productivity was comparable in Gln, Ala-Gln, or Gly-Gln. Substrate utilization and metabolism was affected by the presence of the dipeptides, particularly with Gly-Gln. The specific consumption rates of glucose and six amino acids were reduced and the accumulation of ammonia and lactate was significantly lower.

The replacement of glutamine (2 mM) by pyruvate (10 mM) supported cell growth of several adherent cell lines (MDCK, BHK-21, CHO-K1) in serum-containing and serum-free media, without adaptation for at least 19 passages and with no reduction in growth rate. Even at very low levels of pyruvate (1 mM), MDCK cells grew to confluence without glutamine or accumulation of ammonia. Also glucose uptake was reduced, which resulted in lower lactate production. Amino acid profiles from the cell growth phase for pyruvate medium showed a reduced uptake of serine, cystine, and methionine, an increased uptake of leucine and isoleucine, and a higher release of glycine compared with glutamine medium (Genzel *et al*., 2005).

4.2.3 Amino acids

Amino acids are a class of biomolecules that make a significant contribution to the generation of metabolic energy. The fraction of metabolic energy obtained from amino acids varies greatly with the type of cell and with metabolic conditions.

The transport of amino acids into mammalian cells can be regulated by nutritional, hormonal, or other environmental factors or by changes within cells like transformation. The intracellular or extracellular concentration of amino acids probably has the most profound influence on the efficiency or capacity of transport into animal cells.

The TCA cycle functions as a major route for the synthesis and oxidation of most amino acids and is the primary route through which amino acid carbon flows in the synthesis of other small molecules (*Figure 4.5*).

Amino acids are a vital constituent of all cell culture media. They are normally added as defined components to cell culture medium. The importance of amino acids in synthetic media for *in vitro* growth of mammalian cells has long been recognized, as both a nitrogen donor and a carbon source. Studies on the rates of amino acid uptake have shown generally that glutamine is the most rapidly consumed, followed by lysine, leucine, and isoleucine (Roberts *et al*., 1976). The nutritional requirement for a certain metabolite, however, may also be influenced by the cell population density. For example, serine, cystine, glutamine, and asparagine have been shown to be required at low, but not at high, cell densities (Eagle and Piez, 1962). This occurs in situations when the metabolite is utilized in amounts that exceed the biosynthetic capacity of the cell. The critical population density occurs at the minimum effective intracellular

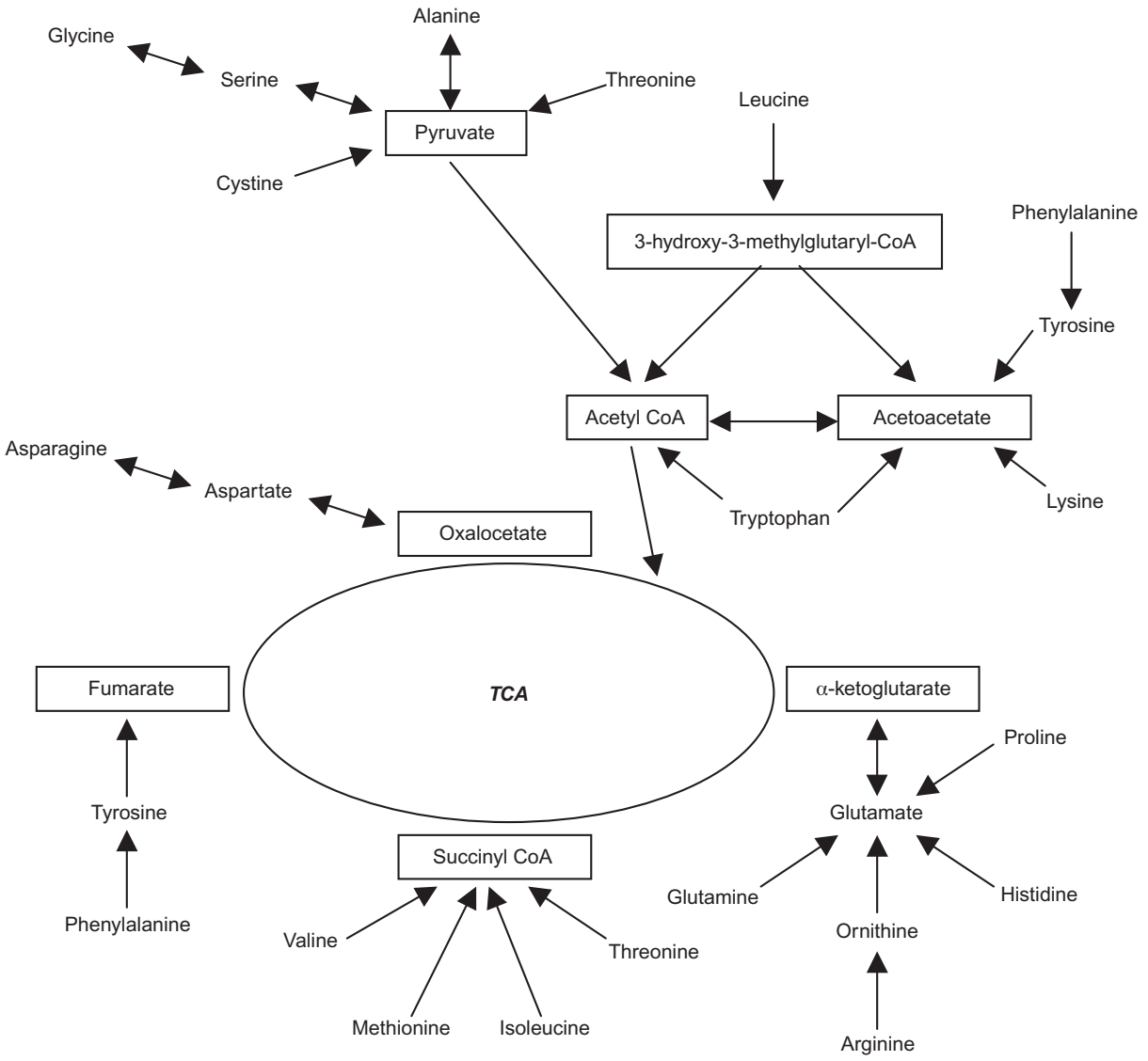


Figure 4.5

Points at which amino acids enter or exit the tricarboxylic acid (TCA) cycle in mammalian cells.

level, before the cells die of the specific deficiency. At high cell densities, however, the cell culture medium may require supplementation with extra amino acids, to prevent their depletion (Doyle and Griffiths, 1998). The uptake of leucine, isoleucine, and methionine is closely related to the cell's position in the cell cycle and is regulated by growth factors (such as insulin) in mammalian cells (Doverskog *et al*., 1997).

Higher eukaryotic cells have lost the ability to synthesize a number of amino acids. These amino acids are generally called essential amino acids, while those that can be synthesized are called non-essential. However, this nomenclature is very misleading for two reasons. First, some of the non-essential amino acids are in fact very essential in that they are required for synthesis of nucleotides (glycine, aspartate, and glutamine). The reason why the ability to synthesize these amino acids has been retained may well be that they are indispensable. Secondly, the capability to synthesize them

may be conditional depending on the nutritional situation, the proliferative status, and cell line-specific properties (Doverskog *et al.*, 1997). The early work of Eagle (Eagle, 1955) demonstrated a need for 12 amino acids to support the proliferation of strain L mouse fibroblasts in medium containing 0.25–2% dialyzed horse serum. Cells would die within 1–3 days in the absence of any one of the 12 amino acids. Glutamine was later added to this list (Eagle, 1959), which also includes arginine, cystine, histidine, isoleucine, leucine, lysine, phenylalanine, methionine, threonine, tryptophan, tyrosine, and valine.

The rates at which these and other amino acids are utilized or produced can vary dramatically between cell lines. The relative concentration of amino acids and serum in the culture medium and other conditions of the culture environment will also influence the rates of utilization or production of specific amino acids.

Amino acids, whose carbon skeleton can be synthesized *de novo* in mammalian cells, include serine, glycine, alanine, aspartate, asparagine, and in principle glutamate and glutamine. The biosynthesis of amino acids is closely linked to the central intermediary metabolism, occurring directly from intermediates of glycolysis or TCA, in one or a few steps. The key enzymes involved are transaminases (Meister, 1955). Although most cells possess glutamate dehydrogenase, it is doubtful if there is any significant net synthesis of glutamate through this enzyme in cultured cells. The allosteric regulation of glutamate dehydrogenase involving up-regulation by GDP (guanosine 5'-diphosphate) and ADP, and inhibition of enzyme activity by GTP and ATP indicates that it has a catabolic function, that is, the enzyme is activated when cells need amino acids for energy production (Mehler, 1982). Glutamine, proline and ornithine are all synthesized from glutamate (Doverskog *et al.*, 1997).

The amino acids that can be synthesized by a cell depend upon the strain-specific profile of biosynthetic enzymes. For example, BHK and CHO cells are capable of glutamine synthesis (Street *et al*, 1993; Neermann and Wagner, 1996), while hybridoma and myeloma cells that do not possess glutamine synthetase are not (Bebbington *et al*., 1992). Another example of a strain-specific difference is the ability to synthesize glycine. Sf9 insect cells and certain CHO cell mutants are reported to be partial glycine auxotrophs (Appling, 1991; Tremblay *et al*., 1992; Chasin *et al*., 1994). The explanation involves the localization of serine hydroxymethyltransferase. This enzyme, which converts serine to glycine and tetrahydrofolate-bound single-carbon units, is present both in the cytoplasm and mitochondria. The mitochondrial isoenzyme activity may be absent in these partial auxotrophs, which are self-supporting in single-carbon units through the cytoplasm enzyme activity, but they need glycine from the medium for protein synthesis. In contrast to mammalian cells, insect cells are much more flexible in their amino acid metabolism (Ferrance *et al*., 1993), some cell lines being capable of synthesizing many more amino acids (Mitsuhashi, 1982), including glutamine, glutamate, and aspartate simultaneously (Öhman *et al.*, 1996).

The capability of synthesis of a certain amino acid may be conditional, and depends on the availability of carbon precursors and nitrogen donors. For example, a glutamine-free medium for mammalian cells may have to

be supplemented with aspartate and/or asparagine (Bebbington *et al*., 1992) to supply intracellular precursors for glutamine biosynthesis and as a source of aspartate and asparagine, the synthesis of which may become limited in a glutamine-free medium. Another example of conditional biosynthesis is the formation of cystine (from methionine, the cystathionine pathway) in Sf9 cells, which appears to be regulated in relation to the proliferative status of the cells (Doverskog *et al.*, 1997).

It has been recognized that certain cells have a specific requirement for an amino acid, for example, serine for lymphoblastoid cells (Birch and Hopkins, 1977). This may be due either to the inability of the cells to make an amino acid, or because the amino acid is decomposed in the medium. The concentration of amino acids usually limits the maximum cell concentration attainable, influences cell survival and growth rate, and can affect the synthesis of certain proteins. A too low concentration of an amino acid can result in rapid depletion from the medium, and is thus ''limiting,'' whereas a too high concentration can be inhibitory.

Branched chain amino acids are consumed particularly rapidly by a number of cell lines including MDCK cells (Butler and Thilly, 1982), human fibroblasts (Lambert and Pirt, 1975), mouse myeloma cells (Roberts *et al*., 1976), and BHK cells (Arathoon and Telling, 1982). Miller *et al*. (1989) found that serine and branched amino acids were more extensively oxidized by hybridoma cells when the specific glutamine utilization rate was low. The sulfur-containing amino acids, methionine and cystine, are also rapidly consumed by cells in culture (Lambert and Pirt, 1975; Butler and Thilly, 1982). It is suggested that a function of glutamine uptake and glutamate formation is to allow cystine uptake by glutamate exchange into the culture medium (Bannai and Ishii, 1988).

Certain amino acids often accumulate in the culture medium during batch growth with surplus glucose and glutamine (Griffiths, 1971; Lanks and Li, 1988; Duval *et al*., 1991; Ljunggren and Häggström, 1992). However, in glutamine-limited fed-batch cultures of myeloma cells the overflow of asparagine, ornithine, proline, alanine, and glutamate is considerably less than in batch cultures. The same situation exists in hybridoma cells (Ljunggren and Häggström, 1995). These results can be explained by the involvement of glutamine and glutamine-derived glutamate, both in the nitrogen transfer reactions and in providing carbon precursors. The results also highlight the close coupling between the amino acid and energy metabolism (Doverskog *et al.*, 1997).

One of the determinants of cell growth and survival in cultures of Sf9 cells is the transport of amino acids across the plasma membrane. Uptake of cystine increases as a function of the cystine concentration in the medium. However, the increased cystine uptake does not lead to an increase in the final cell concentration or in the growth rate. On the contrary, there appears to be a negative influence on cell physiology as more of the energy-yielding substrates, glucose, glutamine, and glutamate are consumed at the higher cystine concentration. This is another example of less tight regulation of metabolism in animal cells (Doverskog *et al.*, 1997).

Alanine, an end product of glutamine metabolism in many mammalian cells (McKeenhan, 1986), is also formed by Sf9 cells. In a normal batch

culture of Sf9 cells with excess glucose and glutamine, alanine is the only overflow metabolite that accumulates to significant concentrations. The use of substrate-limited fed-batch cultures revealed some interesting features of Sf9 metabolism. During glucose limitation alanine formation was totally depressed, while instead ammonium formation was triggered. Glutamine limitation decreased alanine formation somewhat without provoking ammonium formation, and during simultaneous glucose and glutamine limitation, very little overflow metabolism occurred. These results indicate that in Sf9 cells, glucose-derived pyruvate is the carbon precursor for alanine and that glutamine provides the nitrogen. As in hybridoma (Ljunggren and Häggström, 1995) and myeloma cells (Ljunggrenn and Haggstrom 1992), the energy metabolism of Sf9 insect cells becomes more efficient during substrate limitation (Öhman *et al.*, 1996).

Consequently, quantitative amino acid data are important to optimize the formulation of cell culture media. The concentration of many amino acids can be determined and the data used to calculate the rate of utilization or assimilation of the individual amino acids (Doyle and Griffiths, 1998).

4.2.4 Lipids

The oxidation of long-chain fatty acids to acetyl-CoA is a central energy-yielding pathway in many organisms and tissues. In mammalian heart and liver, for example, it provides as much as 80% of the energetic needs under all physiological conditions (Lodish *et al*., 2003).

Lipids have several important functions in animal cells, which include serving as structural components of membranes and as a stored source of metabolic fuel (Griner *et al*., 1993). Eukaryotic cell membranes are composed of a complex array of proteins, phospholipids, sphingolipids, and cholesterol. The relative proportions and fatty acid composition of these components dictate the physical properties of membranes, such as fluidity, surface potential, microdomain structure, and permeability. This in turn regulates the localization and activity of membrane-associated proteins. Assembly of membranes necessitates the coordinate synthesis and catabolism of phospholipids, sterols, and sphingolipids to create the unique properties of a given cellular membrane. This must be an extremely complex process that requires coordination of multiple biosynthetic and degradative enzymes and lipid transport activities.

Saturated fatty acids or unsaturated fatty acids, such as oleic acid (18:1, n-9), can be synthesized by normal mammalian cells that posses elongation and desaturation enzymes (Rosenthal, 1987). However, the polyunsaturated fatty acids of the n-3 and n-6 group, such as linoleic acid (18:2, n-6) or linolenic acid (18:3, n-3), are essential nutrients for animals because they are precursors for the synthesis of eicosanoid hormones such as prostaglandins (Needleman *et al*., 1986).

The enzymes of fatty acid oxidation in mammalian cells are located in the mitochondrial matrix. The fatty acids with chain lengths of 12 or fewer carbons enter the mitochondria without the help of membrane transporters. Those with 14 or more carbons, which constitute the majority of fatty acids obtained in the diet or released from adipose tissue, cannot pass

directly through the mitochondrial membranes: they must first undergo the three enzymatic reactions of the carnitine shuttle. The first reaction is catalyzed by a family of isozymes (different isozymes for fatty acids having short, intermediate, or long carbon chain) present in the outer mitochondrial membrane, the acyl-CoA synthetases, which promote the general reaction:

$$\text{Fatty acid} + \text{CoA} + \text{ATP} \leftrightarrow \text{fatty acyl-CoA} + \text{AMP} + \text{PP}$$

Thus, acyl-CoA synthetases catalyze the formation of a thioester linkage between the fatty acid carboxyl group and the thiol group of coenzyme A to yield fatty acyl-CoA, coupled to the cleavage of ATP to form AMP (adenosine monophosphate) and PP (Pyrophosphate). Fatty acyl-CoA molecules, like acetyl-CoA, are high energy compounds. Fatty acyl-CoA esters formed at the cytosolic side of the outer mitochondrial membrane can be transported into the mitochondria and oxidized to produce ATP, or they can be used in the cytosol to synthesize membrane lipids. The second reaction of fatty acyl-CoA oxidation is a transesterification catalyzed by carnitine acyltransferase I, located in the outer membrane. This enzyme catalyzes the attachment of the fatty acyl-CoA to the hydroxyl group of carnitine to form fatty acyl-carnitine. The fatty acyl-carnitine ester then enters the matrix by facilitated diffusion through the acyl-carnitine/carnitine transporter in the inner mitochondrial membrane. In the third and final step of the carnitine shuttle, the fatty acyl group is enzymatically transferred from carnitine to intramitochondrial coenzyme A by carnitine acyltransferase II. This isoenzyme, located on the inner face of the inner mitochondrial membrane, regenerates fatty acyl-CoA and releases it, along with free carnitine, into the matrix. Carnitine re-enters the intermembrane space via the acyl-carnitine/carnitine transporter. The carnitine-mediated entry process is the rate-limiting step for oxidation of fatty acids in mitochondria and is a regulation point.

Mitochondrial oxidation of fatty acids takes place in three stages. In the first stage (β-oxidation), fatty acids undergo oxidative removal of successive two-carbon units in the form of acetyl-CoA, starting from the carboxyl end of the fatty acyl chain. The fatty acid undergoes a number of metabolic cycles sufficient for the total conversion to acetyl-CoA. In the second stage, the acetyl groups are oxidized to CO_2 in the TCA cycle, which also takes place in the mitochondrial matrix. The third stage consists of oxidative phosphorylation, where the reduced electron carriers NADH and $FADH_2$ donate electrons to O_2 with the concomitant phosphorylation of ADP to ATP. The energy released by fatty acid oxidation is thus conserved as ATP.

Although the role of lipids in cell culture has been rather neglected, it is recognized that lipids are important for cell proliferation in serum-free media. In this respect the importance of serum albumin has been recognized as a carrier of supplemented fatty acids and lipids. Fatty acids are often included in serum-free media to replace the growth-promoting properties of the lipid components of serum. However, the effect of selective fatty acids on cell growth is variable: they can stimulate (Jager *et al*., 1988; Rose and Connolly, 1990; Grammatikos *et al*., 1994), inhibit

(Calder *et al*., 1991), or have no effect on cell growth (Bailey and Dunbar, 1973; Spector *et al*., 1981; Cornwell and Morisaki, 1984).

The regulatory mechanism of cellular uptake of fatty acids appears to be limited and so the composition of the intracellular lipids is likely to reflect the availability of the fatty acids in the medium. This was shown for the CC9C10 hybridoma (Butler *et al*., 1997) and for BHK and CHO cells (Schmid *et al*., 1991). Thus, cells growing in serum-supplemented cultures are likely to attain a fatty acid composition reflecting that of serum, in which the predominant fatty acids are palmitic, stearic, oleic, and linoleic acids at a ratio 2:1:3:1, respectively.

Over 90% of the fatty acid composition of the cells could be accounted for by linoleic, palmitic, oleic, stearic, and arachidonic acids. Minimal quantities of other fatty acids (C8:0, C10:0, C12:0, C14:0, C18:3) were also determined but were less than 10 mol % in control cells and were decreased to less than 5 mol % after one passage of growth in either linoleic or oleic acid (Butler *et al*., 1997).

Linoleic acid has been shown to enhance the proliferation of mouse mammary epithelial cells by metabolism to arachidonic acid, which is a precursor of prostaglandin E_2 (Bandyopadhyay *et al.*, 1987). However, the mechanism of growth promotion of the unsaturated fatty acids in culture may be related to their importance in the synthesis of cellular membranes (Rintoul *et al*., 1978; Rockwell *et al*., 1980), which may have a significant effect on membrane fluidity (Calder *et al*., 1994).

Unsaturated fatty acids (linoleic or oleic acid) have been shown to be essential for hybridomas in serum-free cultures, as they significantly enhance the cell yield and monoclonal antibody (mAb) productivity (Butler and Huzel, 1995; Butler *et al*., 1997). When Butler *et al*. (1999) cultured the murine hybridoma CC9C10 in spinner flasks at high shear rates to determine the effects of fatty acids, they obtained up to threefold enhanced cell yields in 25 μM linoleic acid or 50 μM oleic acid compared with fatty acid-free control cultures. The half-lives of viable cells were 2.38 and 3.63 h, respectively, for cultures containing 25 and 50 μM linoleic acid, compared with the control culture half-life of 1.97 h (Butler *et al*., 1997). At a higher concentration (over 75 μM), they found that cell yields fall below the level of the control cultures. Hexanoic, lauric, margaric, and stearic acids had no effects on cell growth over the concentration range they tested. Arachidonic, linolenic, octanoic, and myristic acids caused a concentration-dependent inhibition of cell growth. Palmitic and decanoic acids enhanced cell growth marginally (9%) but significantly at 25 μM. Linoleic acid (25 μM) enhanced growth more than oleic acid (25 μM), but an equimolar mixture of oleic and linoleic acid (25 μM) stimulated growth more than either fatty acid alone (Butler *et al*., 1999). The most likely mechanism for growth enhancement in hybridoma cells is that the fatty acids are required as components of phospholipids contained in membranes. The effect of growth enhancement was reversible: when cells that had been passaged continuously in the presence of fatty acids were reintroduced into unsupplemented medium, the growth advantage over control cultures was lost.

The effect of linoleic or oleic acid supplementation on mAbs production has been studied. There was a significant initial enhancement of antibody

titers in fatty acid-supplemented cultures but this enhancement effect gradually decreased to insignificance. Subsequent transfer of the cells to fatty acid-free cultures resulted in a transitory state in which higher mAb titers were re-established for cells previously grown in the presence of the fatty acids. The observed enhancement of mAb titer was significantly higher in cultures supplemented with linoleic acid or linoleic/oleic acid mix than in those supplemented with oleic acid alone (Butler *et al*., 1997).

Partial inhibition of glycolysis by saturated fatty acids has been observed in Ehrlich ascites tumor cells. Palmitate and acetate decreased glutamate formation from glutamine (the first step in glutaminolysis) in this cell line, suggesting the possible role of fatty acids as an alternative energy source (Butler *et al*., 1999).

Butler *et al*. (1997) cultivated a murine B-lymphocyte cell line (CC9C19) and a myeloma (SP2/0) in T-flasks in a serum-free medium supplemented with linoleic acid. They determined that, in the presence of linoleic acid, the glutamine consumption rate was significantly lower, and this correlated with a significant decrease in the specific rate of production of ammonia. The glucose consumption rate was slightly lower but the specific rate of production of lactate increased significantly. This can be explained by a change in the formation of glycolytic end-products. The rate of production of alanine was lower in linoleic acid-grown cells. Alanine is produced from pyruvate by transamination with glutamate in response to the need for sequestration of excess metabolic nitrogen. It is likely that the decreased glutamine utilization in linoleic acid-grown cells would lower the level of intracellular glutamate. This would in turn decrease the transamination reaction and cause a greater proportion of pyruvate to be converted to lactate. These changes were reversible on the removal of the fatty acids (Butler *et al*., 1997, 1999).

There may be several mechanisms for these metabolic effects. Unsaturated fatty acids have been shown to directly activate specific enzymes and to induce DNA synthesis and cytokine release from lymphocytes (Karsten *et al*., 1994). The induction of specific protein synthesis may produce the reduction in glutamine metabolism. The increase in the robustness of the fatty acid-grown hybridomas in agitated cultures could be explained by a high incorporation of the available fatty acids into the cellular phospholipids fraction, which is a major structural component of the outer membrane of the cell (Butler *et al*., 1999).

Cell-protecting additives, such as Pluronic® F-68, are commonly included as a media component to reduce cell damage in gas-sparged, agitated cultures. Despite the value of Pluronic® F-68 in protecting cells, there is a potential problem in that Pluronic® may have some cytosolic effects: it may reduce the yield of the producer cell line in culture and there is a possibility of complexation or co-purification with a cell product. The replacement of Pluronic® with fatty acids, as well as being positive for the half-lives and production of cultured cell lines, provides an alternative method to protect producer cells in agitated cultures (Butler *et al*., 1999).

The effect of the unsaturated fatty acids on protein secretion of cultured cells can be dissociated from growth effects. Recombinant protein produc-

tivity from BHK cells seems to be stimulated by unsaturated fatty acids independently of cell growth (Schmid *et al*., 1991).

4.3 Metabolic byproducts

4.3.1 Lactate

Cells *in vivo* convert a proportion of the pyruvate generated by glycolysis into lactic acid, in a reaction catalyzed by the lactate dehydrogenase enzyme. This lactic acid is largely secreted into the blood; some passes into the liver, where it is reoxidized to pyruvate and either further metabolized to CO_2 aerobically or converted to glucose. Much lactate is metabolized to CO_2 by the heart, which is perfused by blood and can continue aerobic metabolism at times when exercising skeletal muscles secrete lactate. In the case of mammalian cells in culture, the lactic acid is accumulated in the medium. Lactate generation requires the use of pH control methods to avoid the direct negative effects of medium acidification on cell growth.

Generally, lactate concentrations below 20 mM are considered not to show any negative effects (*Table 4.1*). There are several actions that can be performed in a culture process to reduce lactate accumulation, and these have already been mentioned.

Lao and Toth (1997) have studied the effects of lactate concentration on the metabolism of a CHO cell line producing a recombinant glycoprotein, cultivated in batch mode with an initial concentration of sodium lactate of 60 mM. They demonstrated a reduction of glucose and glutamine consumption (decreased by 20%) and ammonia and alanine production (decreased by 64% and 70%, respectively), while productivity was not affected. They showed that cultures with added lactate have no lactate production and they hypothesized that inhibition of lactate dehydrogenase was the cause of this decreased lactate production. Inhibition of lactate dehydrogenase prevents the regeneration of NADH to NAD^+ coupled with the pyruvate/lactate conversion, which leads to an accumulation of NADH. This excess of NADH inhibits glycolysis in the cytosol. Increased efficiency of the malate–aspartate shuttle to transport NADH across mitochondrial membrane would be one of the responses from the cells to retain the glycolytic rate. The lactate inhibition of glycolysis also leads to a lower concentration of pyruvate, which may be the cause of a decrease in glutamine consumption. Due to the diminished energy production from lower glycolytic and glutaminolytic rates, with the diversion of

Table 4.1 Influence of different lactate concentrations on cell growth at constant pH (Wagner, 1997)

Lactate (mM)	Growth	Productivity	Reference
< 20	No effect	No effect	Wagner et al. 1988 Miller et al. 1988
20 40	No effect	Inhibition	Glacken et al. 1988
40 60	Slight inhibition	Inhibition	Glacken et al. 1988
60	Inhibition	Inhibition	Glacken 1988

more energy into maintenance of the ion gradient to counter the effects of a hypertonic environment, growth was inhibited under high concentrations of lactate.

On the other hand, Cruz *et al*. (2000) reported that 28 mM lactate reduced the growth of a BHK cell line by 50%, and that lactate was consumed at concentrations above 30 mM. Increased concentrations of lactate reduced cell growth and specific ammonia production but increased specific glutamine and glucose consumption. The effect of lactate was at least partially due to an increase of osmolarity. An increase in lactate from 0 to 60 mM induced a 40% reduction in specific productivity of a recombinant fusion protein secreted by the BHK cells in both stationary and stirred cultures.

The effects of elevated lactic acid concentration on the cell cycle kinetics of hybridoma cell growth and antibody production in batch culture were studied by Kromenaker and Srienc (1994). When 33 mM lactic acid was initially present, the specific growth rate was reduced by 37% and the cell-specific antibody production rate increased by a factor of 2.6 relative to a control culture with no additional lactic acid.

4.3.2 Ammonia

Ammonia (NH_3) and the ammonium ion (NH_4^+) are highly toxic to mammalian cells. *In vivo*, ammonium is secreted by the cells and transported to the mitochondria of hepatocytes, where it is converted into urea via the urea cycle. Urea production occurs almost exclusively in the liver and is the fate of most of the ammonium channeled there. The urea passes into the bloodstream and thus to the kidneys and is excreted into the urine. Mammalian cells in culture secrete ammonium into the culture medium, where its concentration increases gradually because there is no ammonium recycling pathway (Newland *et al*., 1990).

As previously stated, ammonium in cell culture medium is the product of glutamine metabolism and its spontaneous decomposition at 37°C. Negrotti *et al.* (1989) showed that the half-life for glutamine at 37°C and pH 7.2 is only 7 days. The effects of ammonium on cell metabolism are observed from concentrations as low as 2 mM, which is easily reached in culture systems (*Table 4.2*). For example, the spontaneous decomposition of glutamine can result in 0.1 mM ammonia per day (Butler and Spier, 1984). Anchorage-dependent cells grown on microcarriers produce between 2 and 3 mM ammonia after growth in a batch culture (Butler *et al*.,

Table 4.2 Effect of different medium concentrations of ammonium on growth and productivity (Wagner, 1997)

Ammonium (mM)	Growth	Productivity	Reference
< 2	No effect	No effect	Glacken 1988
2 5	Inhibition	No effect	Glacken et al. 1988 McQueen and Bailey 1990 Glacken 1988
5	Inhibition	Inhibition	Glacken 1988

1983; Butler and Spier, 1984), and hybridomas in batch suspension culture produce between 4.5 and 5.5 mM (Reuveny *et al*., 1986).

Ammonium can either perturb the intracellular or intra-organelle pH and electrochemical gradients, or directly interact with enzymes. The toxic effects can be classified into two possibilities, as described below.

(i) **Perturbation of intracellular pH and electrochemical gradients.** At the physiological pH of 7.1–7.5, which is the pH at which most mammalian cell cultures are maintained, only about 1% of the total concentration of ammonia/ammonium is present as ammonia (NH_3), the rest being ammonium (NH_4^+). Ammonia is a small, uncharged, lipophilic molecule, which readily diffuses across cellular membranes. The small percentage of ammonia present in the extra- and intracellular aqueous phases will diffuse across the membranes, thus rapidly equilibrating any transmembrane gradient of ammonia. Ammonium is produced, in the ionic form, inside the mitochondria (*Figure 4.6A*) by the action of glutaminase and glutamate dehydrogenase. The inner mitochondrial membrane is extremely impermeable to ions; however, ammonia readily passes from the mitochondrial matrix into the cytoplasm. This ammonia outflow from the mitochondria leads to a decrease of the pH in the matrix, since a proton is left behind. Ammonia can diffuse out of the cytoplasm into the environment; however, it can be transported back as ammonium by carrier proteins. The consequence of such a cycle is an acidification of the cytoplasm and the mitochondrial matrix, and an alkalinization of the environment. Ammonia/ammonium derived from glutamine decomposition or added externally (Figure 4.6B) will transiently increase the pH of the cytoplasm due to rapid diffusion of ammonia into the cell. This alkalinization is followed by an acidification due to transport of ammonium by carrier proteins. Diffusion of ammonia into the mitochondria and other organelles leads to an increase of the pH inside these compartments. The result is an alkalinization of the cellular environment and of the inside of the organelles, mitochondria included, and an acidification of the cytoplasm. Thus, it is very important to realize that the physiological consequences of adding extracellular ammonium to the medium are very different to those resulting from ammonium produced intracellularly.

(ii) **Ammonium and enzymatic reactions.** Ammonia or ammonium can participate in enzyme reactions and displace equilibria or interact with regulatory sites of enzymes. The key enzyme of the glycolytic pathway, phosphofructokinase, as well as α-ketoglutarate dehydrogenase, an enzyme of the TCA cycle, have been reported to be activated by ammonium (Uyeda and Racker, 1965; Parmeggiani *et al*., 1966). Thus, elevated ammonium concentrations could lead to a high rate of glycolysis and lactate production and a reduced TCA cycle activity. Other enzymes affected by ammonium concentration are those involved in the glycosylation of proteins. Gramatikos *et al*. (1998) have reported that the intracellular content of UDP-N-acetylhexosamines (UDP-GNAc) is substantially elevated under high am-

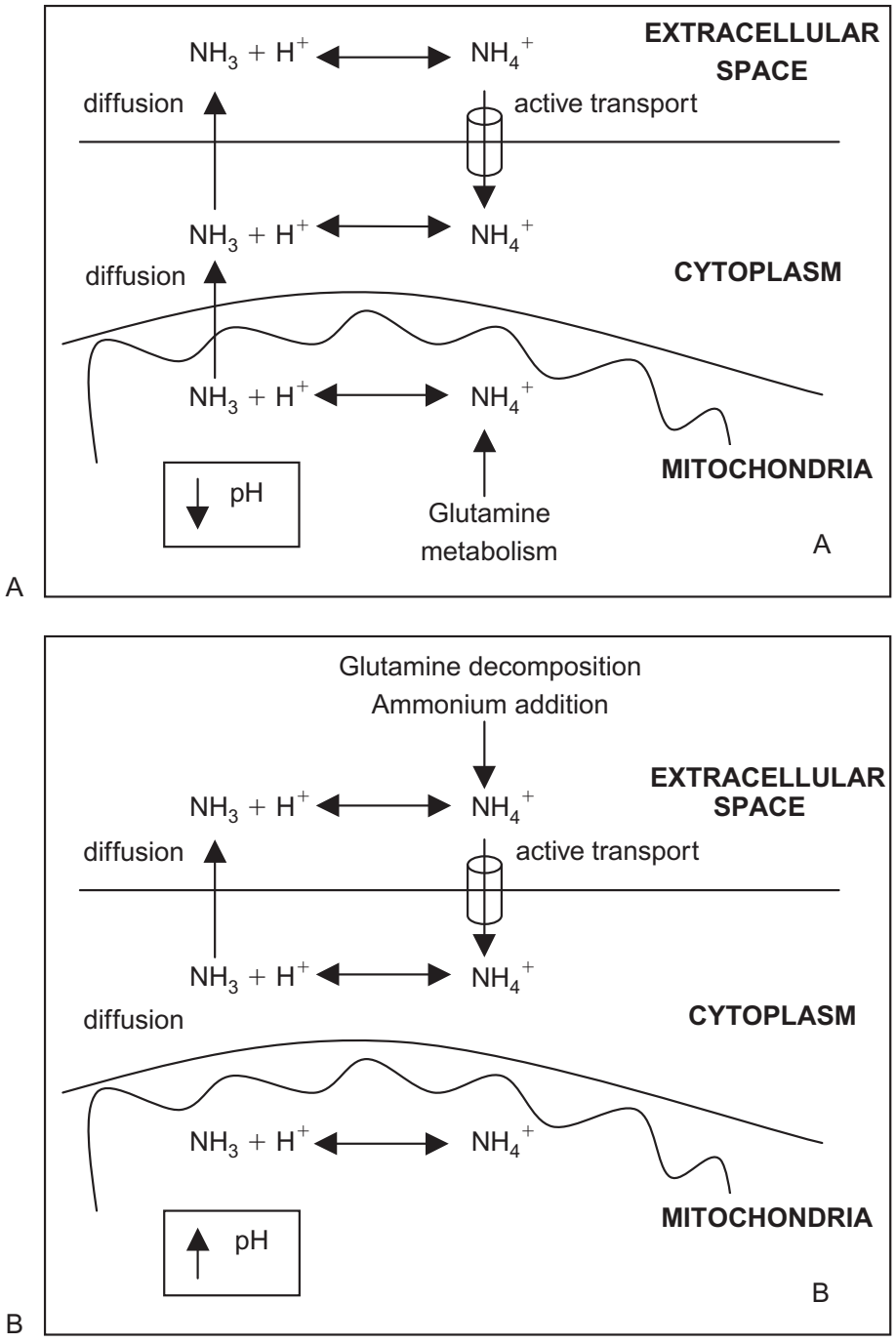


Figure 4.6

Effects of ammonium on mammalian cell cultures. (A) Effects of ammonium generated by glutamine metabolism. (B) Effects of ammonium generated by glutamine decomposition or by ammonium addition in the culture medium.

monium concentrations in the medium. This may lead to an increase in the antennarity of the N-linked oligosaccharides of glycoproteins, or a decrease in terminal sialylation (Yang and Butler, 2002) (see Chapter 6).

The toxic effects produced by ammonium are: (i) enzymatic inhibition (in glycolysis, TCA cycle, glutaminolysis, and PPC); (ii) perturbation of the transcellular ionic gradient; (iii) intracellular pH modification; (iv) increase of UDP-GNAc synthesis, with concomitant glycolysis modification and affecting the quality of the recombinant protein; and (v) increase in the alanine secretion.

As shown schematically in *Figure 4.7*, an increase of the UDP-GNAc concentration in the cytoplasm leads to elevated transport of UDP-GNAc into the Golgi vesicle, by means of the sugar nucleotide transport system I. This higher concentration of UDP-GNAc, however, inhibits the sugar nucleotide transport system II that is responsible for CMP-NANA import to the Golgi, and a higher CMP-NANA concentration inhibits in turn, via feedback inhibition, the UDP-*N*-acetylglucosaminyl epimerase that catalyzes the first step of UDP-NANA synthesis. This block, together with the inhibition of the transport system, reduces CMP-NANA concentration, resulting in a decrease of sialylation.

It was reported by Yang and Butler (2002) that ammonia caused an increase in the antennarity and a decrease in the sialylation of glycans of recombinant EPO in CHO cells. Gawlitzek *et al*. (1998) have studied the final carbohydrate structure of a human interleukin (IL)-2 mutant glycoprotein, named IL-Mu6. Furthermore, Valley *et al*. (1999) demonstrated that about 60–80% of N-acetylated sugars in N-glycan structures contained ^{15}N, provided by $^{15}NH_4Cl$ used as a supplement in the culture medium. This indicates that ammonium is used as a building block during synthesis of the carbohydrate structures expressed in cultivated mammalian cells. Gawlitzek *et al*. (2000) cultured a BHK-21 recombinant clone in a continuously perfused double membrane bioreactor. In the presence of 15 mM exogenously added NH_4Cl, a significant and reproducible increase in tri- and tetra-antennary oligosaccharides (45% of total) was detected in the secretion product. They also studied the effect of ammonium on the glycosylation pattern of the recombinant immunoadhesin tumor necrosis factor-IgG (TNFR-IgG) produced by CHO cells. They demonstrated that as ammonium increased from 1 to 15 mM, a concomitant decrease of up to 40% was observed in terminal galactosylation and sialylation of the molecule. Gawlitzek *et al*. (1999) cultivated recombinant CHO cells producing an immunoadhesin glycoprotein (GP1-IgG) under controlled conditions in the presence of different ammonium N-Glycans synthesized in the presence of $^{15}NH_4Cl$ revealed an N-glycan-dependent increase in mass to charge ratio of 2.5–4.8 Da. A 60–70% of the total nitrogen contained in the monosaccharides was ^{15}N, which suggested that $^{15}NH_4^+$ was incorporated into GlcNAc and N-acetylneuraminic acid as proposed earlier (Ryll *et al*., 1994). Studies of secreted EPO from CHO recombinant cells during cultivation in a medium supplemented with NH_4Cl revealed that the presence of ammonium caused a significant increase in the heterogeneity of the glycoforms, as shown by the isoelectric point, which increased from a range of 4.06–4.67 in the control culture to 4.18–6.05, when ammonium was added to the culture medium. A shift in molecular weight, from 33 000–39 000 in the control culture to 27 000–37 000 in the ammonium-supplemented medium, was further evidence for the increased heterogeneity. Cell growth was inhibited above a culture concentration of

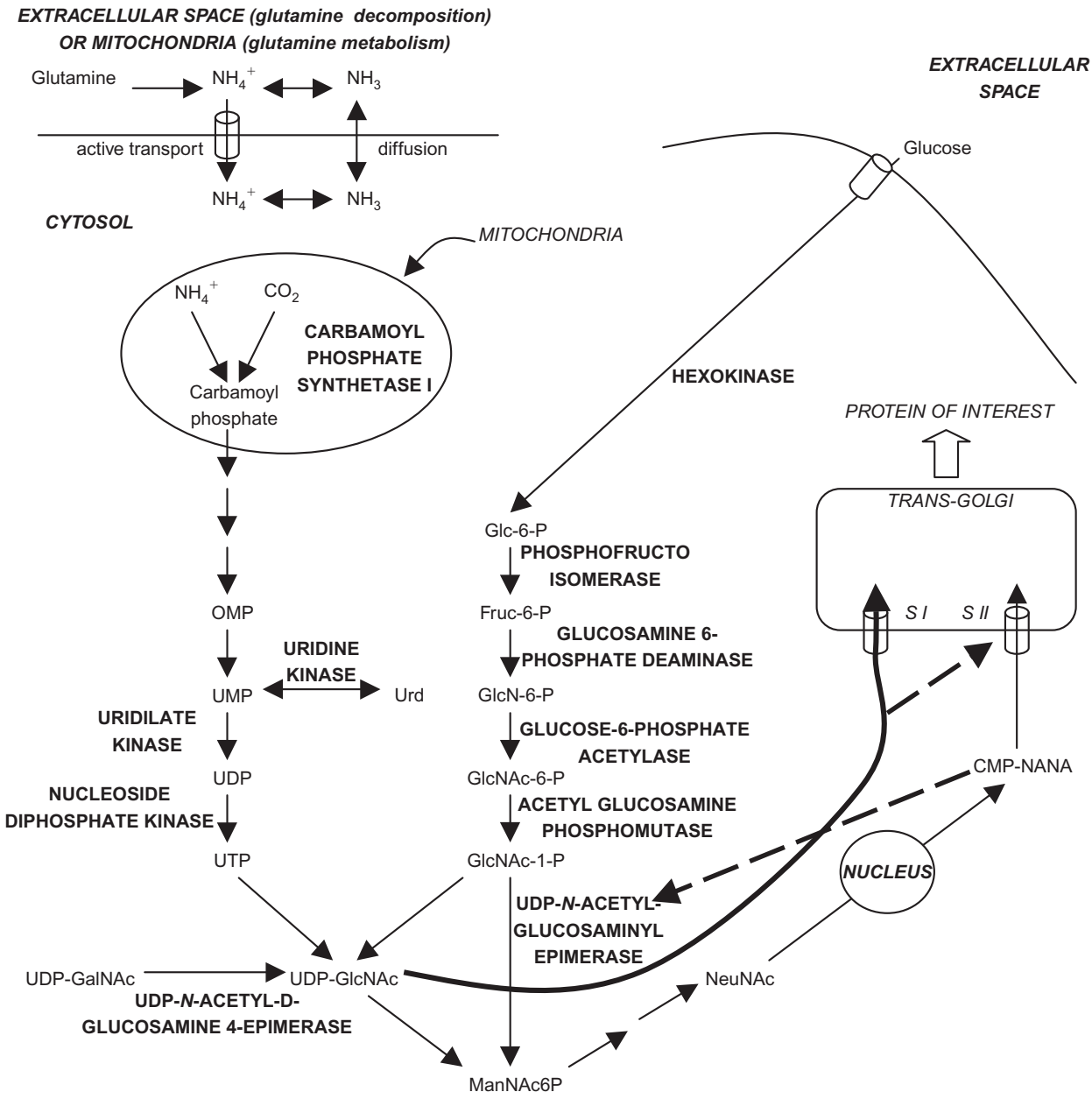


Figure 4.7

Effects of increased ammonium concentrations on the glycosylation of proteins. OMP, orotidine monophosphate; UMP, uridine monophosphate; UDP, uridine diphosphate; UTP, uridine triphosphate; UDP GlcNAC, UDP *N* acetylglucosamine; UDP GalNAc, UDP *N* acetylgalactosamine; ManNAc6P, mannose *N* acetyl 6 phosphate; NeuNAc, N acetylsialic acid; CMP NANA, cytosine monophosphate *N* acetylneuraminic acid; S I, sugar nucleoside transport system I; S II, sugar nucleoside transport system II; Glc 6 P, glucose 6 phosphate; Fruc 6 P, fructose 6 phosphate; GlcN 6 P, glucosamine 6 phosphate; GlcNAc 6 P, *N* acetylglucosamine 6 phosphate; GlcNAc 1 P, *N* acetylglucosamine 1 phosphate. The dashed arrows indicate the influence of the increased ammonium concentration.

5 mM NH_4Cl, whereas the specific production of EPO increased with the addition of NH_4Cl above this level. At 10 mM NH_4Cl, the final cell density after 4 days in culture was significantly lower but the final yield of EPO was significantly higher. This appeared to be due to continued protein production after cell growth had ceased. The metabolic effects of added NH_4Cl included higher specific consumption rates of glucose and glutamine and an increased rate of production of alanine, glycine, and glutamate (Yang and Butler, 2000).

Sun and Zhang (2001b) showed that an increase of the initial ammonia concentration resulted in decreased cellular yields with respect to glucose, glutamine, and other consumed amino acids. In a batch culture with an initial ammonia concentration of 5.66 mM, the cellular yields from glucose and glutamine reduced by 78% and 74%, respectively, compared to that with ammonia at an initial concentration of 0.21 mM. The yields of cells with respect to other consumed amino acids also decreased by 50–70%. The metabolic pathways were altered in the cultures with the higher ammonia concentrations. The glucose consumption was more prone to form lactate. For glutamine metabolism, the reaction of glutamate to α-ketoglutarate catalyzed by the glutamate dehydrogenase was inhibited by ammonia, and that by the glutamate amino transferase was facilitated. However, the yields of glutamate to glutamine decreased with the increase of ammonia concentrations, showing that the reaction of glutamate to α-ketoglutarate was inhibited by ammonia as a whole. Lao and Toth (1997) reported a decrease in alanine production. While aspartate consumption increased sixfold, glutamate changed from consumption to low-level production and higher consumption again with added ammonium concentrations. Cells consumed more aspartate and glutamine at high ammonium concentrations. There was also a decrease in TCA intermediates, for example, malate (Lao and Toth, 1997).

Chen *et al*. (2005) investigated the addition of amino acids to the growth media for CHO cell cultures as a means of mitigating the negative effects of ammonium. Threonine, proline, and glycine additions enhanced CHO cell growth and recombinant protein levels. Furthermore, the addition of these amino acids positively impacted important metabolic parameters, including glucose consumption, lactate production, glutamine utilization, and final ammonium levels. Additionally, threonine, proline, and glycine increased the level of α-2,3-linked sialic acid, galactose-β-1,4-N-acetylglucosamine, and α-2,6-linked sialic acid residues in recombinant tissue plasminogen activator (tPA). Thus, these amino acids can be used to mitigate some of the toxic effects of ammonium on cell growth, recombinant protein productivity, and protein quality.

4.4 Factors affecting cell metabolism

There are several factors that have a large influence on metabolic pathways. Among the external factors, the most important are the dissolved oxygen and carbon dioxide concentration, the pH, and the temperature of the culture. These factors are discussed in Chapter 2, but here their effects on metabolic pathways are described.

4.4.1 Oxygen requirements

The effects of dissolved oxygen on mammalian cell growth and metabolism have been studied extensively. It has been established that anaerobic conditions can severely depress cell growth. At high oxygen concentrations, on the other hand, oxidative damage is observed. Between these limits, there exists an optimal dissolved oxygen range suitable for proliferation of mammalian cells. Generally, a dissolved oxygen range between 30% and 60% air saturation is accepted to be optimal for cell growth, although the sensitivity of cells to extreme dissolved oxygen levels is known to vary. Each cell line seems to have a different optimal dissolved oxygen range. It has been shown that established cell lines are more tolerant to high dissolved oxygen levels. Lymphoblastoid cells, for instance, achieved their highest cell yield and growth rates at high dissolved oxygen concentrations (100%).

WI-38 cells grown at dissolved oxygen values between 16% and 85% air saturation had similar growth parameters, such as population density, glucose utilization, and lactate production. The growth rate decreased greatly and metabolic rates of glucose and lactate increased four- to six-fold when the dissolved oxygen was lowered to 5%. Similar increases in glucose utilization and lactate production at low dissolved oxygen have been reported for other cell lines. Less is known about the effects of dissolved oxygen on glutamine metabolism. An increase in glutamine consumption rate at very low and very high oxygen concentrations has been reported. Effects of oxygen were also reported on ammonia production from glutamine (Ozturk and Palssom, 1990).

The murine hybridoma CC9C10 was grown at steady state, under serum-free conditions in continuous culture, at dissolved oxygen concentrations in the range of 10–150% of air saturation. There was a requirement for cellular adaptation at each stepwise increase in dissolved oxygen. Adaptation to a dissolved oxygen concentration of 100% was associated with an increase in the specific activities of glutathione peroxidase (18-fold), glutathione S-transferase (11-fold), and superoxide dismutase (6-fold), which are all known antioxidant enzymes. At dissolved oxygen concentrations above 100%, the activities of glutathione peroxidase and glutathione S-transferase decreased, possibly as a result of inactivation by reactive oxygen radicals. The increase in dissolved oxygen concentration caused changes in energy metabolism. The specific rate of glucose uptake increased at higher dissolved oxygen concentrations, with a higher proportion of glucose metabolized anaerobically. It was shown that the flux of glucose through glycolysis and the pentose phosphate pathway increased, whereas the flux through the tricarboxylic acid cycle decreased at high dissolved oxygen concentrations (Jan *et al*., 2000).

Acute exposure of Chinese hamster ovary (CHO) cells or human bladder cancer MGH-U1 cells to hypoxia plus low pH (6.5–6.0), was cytotoxic in a time- and pH-dependent manner. Inhibition of glycolysis by incubation of CHO cells under hypoxic conditions in the absence of glucose at pH 7.0 led to a larger fall in cellular ATP and energy charge. A decrease in energy charge of the cells may contribute to loss of viability, but additional mechanisms appear to be involved (Rotin *et al*., 1986).

Activities of enzymes involved in glycolysis, the pentose phosphate pathway, the TCA cycle, and glutaminolysis have been determined in the mouse myeloma SP2/0.Ag14, when the cells were grown on IMDM medium with 5% serum in steady-state chemostat culture under different levels of oxygenation. The specific rates of glucose consumption and lactate production were highest at low oxygen supply. Under low oxygen conditions, the specific production of ammonia increased and the consumption pattern of amino acids showed large changes compared with the control culture. The levels of glycolytic enzymes were elevated under conditions of low oxygen supply (Vriezen *et al*., 1997).

Not only does the oxygen level affect metabolism, but also metabolism affects oxygen uptake rates, making a tight control of oxygen concentration necessary when the composition of the culture medium is changed.

4.4.2 Carbon dioxide

Carbon dioxide is one of the end products of mammalian cell metabolism, produced in different metabolic steps. In some reactions, carbon dioxide can be used as a substrate, such as the carboxylation of pyruvate to oxaloacetic acid. The importance of carbon dioxide is also related to its generalized use in cell culture technology for pH regulation, in equilibrium with medium bicarbonate. The influence of carbon dioxide generation by cell metabolism can be especially relevant in industrial applications, operating with systems optimized to provide an efficient oxygen supply and to reach high cell densities. In such systems, the metabolically produced carbon dioxide may increase, with an elevated partial pressure, which can lead to reduced cell viability, decreased specific production rates, and polysialylation rates (Zanghi *et al*., 1999).

4.4.3 Temperature

Standard temperatures are usually defined for every known cell line, for example, mammalian cell lines are usually cultured at 37°C and insect cell lines at 28°C. However, studies have demonstrated that temperature modifications can lead to changes in certain parameters, such as increased productivity of recombinant proteins or decreased byproduct generation.

Several studies have shown environmental control strategies to affect protein glycosylation and carbon metabolism, as well as cell growth and cell death. Experiments suggest that reduced culture temperature results in higher viability and shear resistance, decreased specific growth rate, limited release of waste products, and reduced glucose/lactate metabolism. There are several reports on the effects of the culture temperature on cells producing recombinant proteins (Bollati-Fogolin *et al*., 2005). In some cases reduced temperatures were associated with improved cellular productivity, and prolonged cellular viability in culture (Rodriguez *et al*., 2005). However, Weidemann *et al*. (1994) described that a temperature below 37°C reduced the cell growth and glucose consumption of a recombinant BHK cell line but did not affect the cellular productivity of recombinant antithrombin III.

4.4.4 pH

Ideally, the pH should be near 7.4 at the initiation of a culture and remain above a value of 7.0 during the culture, although some hybridomas appear to prefer lower pH. A pH below 6.8 is usually inhibitory to cell growth. Factors affecting the pH stability of the medium are buffer capacity, volume of headspace, and glucose concentration.

As described in Chapter 2, the usual normal buffer system in culture media is the carbon dioxide-bicarbonate system, analogous to that in the blood. Modifications in the medium pH produce changes to the intracellular pH value, with modifications to enzyme activities.

4.5 Conclusions

Mammalian cell culture is a technology used for the production of recombinant proteins of therapeutic use, as they can secrete proteins with post-translational modifications similar to those present in human proteins. The most important advantages of this capacity of the mammalian cell lines are that they secrete a protein with the similar characteristics to the original protein, so that the protein can be used for human treatment without generating immunological responses.

As the productivity of mammalian cell lines is lower compared with that of other production systems (bacteria, insect cells, etc.), it is desirable to improve the productivity per cell or the total cell yield per unit volume of culture. An increase in the specific productivity could involve enhancement at the genetic level by gene amplification or by the addition of an inducer to enhance the transcription of a gene. However, knowledge of mammalian cell metabolism helps in the development of strategies for productivity enhancement, as changes in the culture conditions can affect both metabolism and productivity.

The cell yield can be improved by a full understanding of the physicochemical environment of the cells during growth and maintenance. It is now becoming clear that cells in culture are highly adaptable and may be maintained under conditions that are substantially different from their normal environment *in vivo*. Intracellular enzyme activity levels can change considerably and such changes can alter fluxes through metabolic pathways, which may reflect the optimal utilization of the nutrients made available in the culture medium (Crabtree and Newsholme, 1987; Glacken, 1988; Butler *et al*., 1989).

Thus, knowledge of cell physiology is an important prerequisite for further directed metabolic optimization and the investigation of associated cellular phenomena.

References

Appling DR (1991), Compartmentation of folate mediated one carbon metabolism in eukaryotes, FASEB J. 5:2645 2651.

Arathoon WR, Telling CR (1982), Uptake of amino acid and glucose by BHK 21 clone 13 suspension cells during cell growth, Dev. Biol. Stand. 50:145 154.

Bailey JM, Dunbar LM (1973), Essential fatty acid requirements of cells in tissue

culture: a review, Exp. Mol. Pathol. 18:142 161.
Bandyopadhyay GK, Imagawa W, Wallace D, Nandi S (1987), Linoleate metabolites enhance the *in vitro* proliferative response of mouse mammary epithelial cells to epidermal growth factor, J. Biol. Chem. 262:2750 2756.
Bannai S, Ishii T (1988), A novel function of glutamine in cell culture: utilization of glutamine for the uptake of cystine in human fibroblasts, J. Cell. Physiol. 137:360 366.
Bebbington CR, Renner G, Thomson S, King D, Abrams D, Yarranton GT (1992), High level expression of a recombinant antibody from myeloma cells using a glutamine synthetase gene as an amplifiable selectable marker, Biotechnology 10:169 175.
Bell SL, Bushell ME, Scott MF, Wardell JN, Spier RE, Sanders PG (1992), Genetic modification of hybridoma glutamine metabolism: physiological consequences. In: Proceedings of the 11th ESACT Meeting, Brighton, UK, pp. 180 182.
Bell SL, Bebbington C, Scott MF, Wardell JN, Spier RE, Bushel ME, Sanders PG (1995), Genetic engineering of hybridoma glutamine metabolism, Enzyme Microb. Technol. 17:98 106.
Birch JR, Hopkins DW (1977), The serine and glycine requirements of cultured human lymphocyte lines. In: Acton RT, Lynn JD (Eds), Cell Culture and its Applica tions, Academic Press, New York, pp. 503 511.
Bollati Fogolin M, Forno G, Nimtz M, Conradt HS, Etcheverrigaray M, Kratje R (2005), Temperature reduction in cultures of hGM CSF expressing CHO cells: effect on productivity and product quality, Biotechnol. Prog. 21:17 21.
Burant CF, Takeda J, Brot Laroche E, Bell GI, Davidson NO (1992), Fructose transporter in human spermatozoa and small intestine is GLUT5, J. Biol. Chem. 267:14523 14526.
Butler M (2004), Animal Cell Culture & Technology, 2nd Ed., BIOS Scientific Publishers, New York.
Butler M, Christie A (2004), Adaptation of mammalian cells to non ammoniagenic media, Cytotechnology 15:87 94.
Butler M, Huzel N (1995), The effects of fatty acids on hybridoma cell growth and antibody productivity in serum free cultures, J. Biotechnol. 39:165 173.
Butler M, Spier RE (1984), The effects of glutamine utilization and ammonia pro duction on the growth of BHK cells in microcarrier cultures, J. Biotechnol. 1:187 196.
Butler M, Thilly WG (1982), MDCK microcarrier cultures: seeding density effects and amino acid utilization, In Vitro 18:213 219.
Butler M, Imamura T, Thomas WG, Thilly M (1983), High yields from microcarrier cultures by medium perfusion, J. Cell. Sci. 61:351 363.
Butler M, Huzel N, Barnabé N (1997), Unsaturated fatty acids enhance cell yields and perturb the energy metabolism of an antibody secreting hybridoma, Biochem. J. 322:615 623.
Butler M, Huzel N, Barnabé N, Gray T, Bajno L (1999), Linoleic acid improves the robustness of cells in agitated cultures, Cytotechnology 30:27 36.
Butler, M. and Jenkins, H. (1989), Nutritional aspects of growth of animal cells in culture. J. Biotechnol. 12, 97 110.
Calder PC, Bond JA, Bevan SJ, Hunt SV, Newsholme EA (1991), Effect of fatty acids on the proliferation of concanavalin A stimulated rat lymph node lymphocytes, Int. J. Biochem. 23:579 588.
Calder PC, Yaqoob P, Harvey DJ, Watts A, Newsholme EA (1994), Incorporation of fatty acids by concanavalin A stimulated lymphocytes and the effects on fatty acid composition and membrane fluidity, Biochem. J. 300:509 518.
Carruthers A (1990), Facilitated diffusion of glucose, Physiol. Rev. 70:1135 1176.
Chasin LA, Feldman A, Konstam M, Urlaub G (1994), Reversion of a Chinese hamster

cell auxotrophic mutant, Proc. Natl Acad. Sci. U S A 71:718 722.
Chen J, Sun X, Zhang Y (2005), Growth and metabolism of marine fish Chinook salmon embryo cells: response to lack of glucose and glutamine, Biotechnol. Lett. 27:395 401.
Chen P, Harcum SW (2005), Effects of amino acids additions on ammonium stressed CHO cells, J. Biotechnol. 117:277 286.
Christie A, Butler M (1994), Glutamine based dipeptides are utilized in mammalian cell culture by extracellular hydrolysis catalyzed by a specific peptidase, J. Biotechnol. 37:277 290.
Cornwell DG, Morisaki N (1984), Fatty acid paradoxes in the control of cell prolifera tion: prostaglandins, lipid peroxides and cooxidation reactions, In: Pryor WA (Ed.), Free Radicals in Biology VI, Academic Press, New York, pp. 95 147.
Crabtree B, Newsholme EA (1987), A systematic approach to describing and analyzing metabolic control systems, Trends Biochem. Sci. 12:6 12.
Craik JD, Elliot KRF (1979), Kinetics of 3 O methyl D glucose transport in isolated rat hepatocytes, Biochem. J. 182:503 508.
Cruz HJ, Freitas CM, Alves PM, Moreira JL, Carrondo MJT (2000), Effects of ammonia and lactate on growth, metabolism, and productivity of BHK cells, Enzyme Microb. Technol. 27:43 52.
Donelly M, Scheffler IE (1976), Energy metabolism in respiration deficient and wild type Chinese hamster fibroblasts in culture, J. Cell. Physiol. 18:39 52.
Doverskog M, Ljunggren J, Öhman L, Häggström L (1997), Physiology of cultured animal cells, J. Biotechnol. 59:103 115.
Doyle A, Griffiths JB (1998), Cell and Tissue Culture: Laboratory Procedures in Biotechnology, John Wiley & Sons, Chichester, UK.
Duval D, Demangel C, Munier Jolain K, Miossec S, Geahel I (1991), Factors control ling cell proliferation and antibody production in mouse hybridoma cells: I. Influence of the amino acid supply, Biotechnol. Bioeng. 38:561 570.
Duval D, Demangel C, Miossec S, Geahel I (1992), Role of metabolic waste products in the control of cell proliferation and antibody production by mouse hybridoma cells, Hybridoma 11:311 322.
Eagle H (1955), Nutrition needs of mammalian cells in tissue cultures, Science 122:501 504.
Eagle H (1959), Amino acid metabolism in mammalian cell cultures, Science 130: 432 437.
Eagle H, Piez K (1962), The population dependent requirement by cultured mamma lian cells for metabolites which they can synthesize, J. Exp. Med. 116:29 43.
Elliott WH, Elliott DC (2001), Biochemistry and Molecular Biology, 2nd Ed., Oxford University Press, New York.
Ferrance JP, Goel A, Ataai MM (1993), Utilization of glucose and amino acids in insect cell cultures: quantifying the metabolic flows within the primary pathways and medium development, Biotechnol. Bioeng. 42:697 707.
Fitzpatrick L, Jenkins HA, Butler M (1993), Glucose and glutamine metabolism of a murine B lymphocyte grown in batch culture, Appl. Biochem. Biotechnol. 43: 93 116.
Gawlitzek M, Valley U, Wagner R (1998), Ammonium ion and glucosamine dependent increases of oligosaccharide complexity in recombinant glycoproteins secreted from cultivated BHK 21 cells, Biotechnol. Bioeng. 57:518 528.
Gawlitzek M, Papac DI, Sliwkowski MB, Ryll T (1999), Incorporation of 15N from ammonium into the N linked oligosaccharides of an immunoadhesin glycoprotein expressed in Chinese Hamster Ovary cells, Glycobiology 9:125 131.
Gawlitzek M, Valley U, Wagner R (2000), Ammonium alters N glycan structures of recombinant TNFR IgG: degradative versus biosynthetic mechanisms. Biotech nol. Bioeng. 68:637 646.

Genzel Y, Ritter JB, Konig S, Alt R, Reichl U (2005) Substitution of glutamine by pyruvate to reduce ammonia formation and growth inhibition of mammalian cells. Biotechnol. Prog. 21:58 69.
Glacken MW (1988), Catabolic control of mammalian cell culture, Biotechnology 6:1941 1950.
Glacken MW, Adema E, Sinskey AJ (1988), Mathematical descriptions of hybridoma culture kinetics I. Initial metabolic rates, Biotechnol. Bioeng. 28:1376 1389.
Gould GW, Thomas HM, Jess TJ, Bell GI (1991), Expression of human glucose transporters in *Xenopus oocytes*. Kinetic characterization and substrate specifici ties of the erythrocyte, liver, and brain isoforms, Biochemistry 30:5139 5145.
Grammatikos SI, Subbaiah PV, Victor TA, Miller WM (1994), Diverse effects of essential (n 6 and n 3) fatty acids on cultured cells, Cytotechnology 15:31 50.
Gramatikos SI, Valley U, Nimtz M, Conrad HS, Wagner R (1998), Intracellular UDP *N* acetylhexosamine pool affects *N* glycan complexity: a mechanism of ammo nium action on protein glycosylation, Biotechnol. Prog. 14:410 419.
Griffiths B (2000), Scaling up of animal cell cultures, In: John RW (Ed.), Animal Cell Culture, 3rd Ed., Masters, Oxford University Press, pp. 19 63.
Griffiths JB (1971), The effect of medium changes on the growth and metabolism of the human diploid cell, WI 38, J. Cell Sci. 8:43 52.
Griner RD, Aleo MD, Schnellmann RG (1993), The role of short chain fatty acid substrates in aerobic and glycolytic metabolism in primary cultures of renal proximal tubule cells, In Vitro Cell Dev. Biol. Anim. 29A:649 655.
Gstraunthaler G, Seppi T, Pfaller W (1999), Impact of culture conditions, culture media volumes and glucose content on metabolic properties of renal epithelial cell cultures. Are renal cells in tissue culture hypoxic?, Cell. Physiol. Biochem. 9: 150 172.
Haggstrom L (2000), Animal cell metabolism In: Spier R (Ed.), Encyclopedia of Cell Technology, Wiley & Sons, New York, pp. 392 411.
Hassell T, Butler M (1990), Adaptation to non ammoniagenic media and selective substrate feeding lead to enhanced yields in animal cell culture, J. Cell Sci. 96: 501 508.
Hassell T, Gleave S, Butler M (1991), Growth inhibition in animal cell culture: the effect of lactate and ammonia, Appl. Biochem. Biotechnol. 30:29 41.
Jager V, Lehmann J, Friedl P (1988), Serum free growth medium for the cultivation of a wide spectrum of mammalian cells in stirred bioreactors, Cytotechnology 1:319 329.
Jan DCH, Petch DA, Huzel N, Butler M (2000), The effect of dissolved oxygen on the metabolic profile of a murine hybridoma grown in serum free medium in continuous culture, Biotechnol. Bioeng. 54:153 164.
Kahn BB (1992), Facilitative glucose transporters. Regulatory mechanisms and de regulation in diabetes, J. Clin. Invest. 89:1367 1374.
Karsten S, Schafer G, Schauder P (1994), Cytokine production and DNA synthesis in human peripheral lymphocytes in response to palmitic, stearic, oleic and linoleic acids, J. Cell Physiol. 161:15 22.
Kayano T, Fukumoto H, Eddy RL, Fan YS, Byers MG, Shows TB, Bell GI (1988), Evidence for a family of human glucose transporter like proteins. Sequence and gene localization of a protein expressed in fetal skeletal muscle and other tissues, J. Biol. Chem. 263:15245 15248.
Kromenaker SJ, Srienc F (1994), Effect of acid lactic on the kinetics of growth and antibody production in a murine hybridoma: secretion patterns during the cell cycle, J. Biotechnol. 34:13 34.
Lambert J, Pirt SJ (1975), The quantitative requirements of human diploid cells (strain MRC 5) for amino acids, vitamins and serum, J. Cell Sci. 17:397 41.
Lanks KW, Li PW (1988), End products of glucose and glutamine metabolism by

cultured cell lines, J. Cell Physiol. 135:151 155.
Lao M, Toth D (1997), Effects of ammonium and lactate on growth and metabolism of a recombinant Chinese Hamster Ovary cell culture, Biotechnol. Prog. 13: 688 691.
Ljunggren J, Häggström L (1992), Glutamine limited fed batch culture reduces the overflow metabolism of amino acids in myeloma cells, Cytotechnology 8:45 56.
Ljunggren J, Häggström L (1995), Specific growth rate as a parameter for tracing growth limiting substances in animal cell cultures, J. Biotechnol. 42:163 175.
Lodish H, Scott MP, Matsudaria P, Darnell J, Zipursky L, Kaiser CA, Berk A, Krieger M (2003), Molecular Cell Biology, 5th Ed., Freeman and Company, New York.
McDermott RH, Butler M (1993), Uptake of glutamate, not glutamine synthetase, regulates adaptation of mammalian cells to glutamine free medium, J. Cell Sci. 104:51 58.
McKeehan WL (1986), Glutaminolysis in animal cells, In: Morgan MM (Ed.), Carbo hydrate Metabolism in Cultured Cells, Plenum, New York, pp. 111 150.
McQueen A, Bailey E (1990), Effect of ammonium ion and extracellular pH on hybridoma cell metabolism and antibody production, Biotechnol. Bioeng. 35:897 907.
Maranga L, Goochee CF (2006), Metabolism of PER.C6® cells cultivated under fed batch conditions at low glucose and glutamine levels, Biotechnol. Bioeng. 94: 139 150.
Mehler AH (1982), Amino acid metabolism I: General Pathways, In: Devlin TM (Ed.), Textbook of Biochemistry with Clinical Correlations, 2nd Ed. Wiley, New York, pp. 437 452.
Meijer JJ, van Dijken JP (1995), Effects of glucose supply on myeloma growth and metabolism in chemostat culture, J. Cell. Physiol. 162:191 198.
Meister A (1955), Transamination, In: Nord FF (Ed.), Advances in Enzymology, Interscience, New York, pp. 185 246.
Miller WM, Wilke CR, Blanch HW (1988), Transient response of hybridoma cells to lactate and ammonia pulse and step changes in continuous culture, Bioprocess Eng. 3:113 122.
Miller WM, Wilke CR, Blanch HW (1989), Transient response of hybridoma cells to nutrient additions in continuous culture: I. Glucose pulse and step changes, Biotechnol. Bioeng. 33:487 499.
Mitsuhashi J (1982), Determination of Essential Amino Acids for Insect Cell Lines, Academic Press, New York.
Needleman P, Turk J, Jakschik BA, Morrison AR, Lefkowith JB (1986), Arachidonic acid metabolism, Annu. Rev. Biochem. 55:69 102.
Neerman J, Wagner R (1996), Comparative analysis of glucose and glutamine metabo lism in transformed mammalian cell lines, insect and primary liver cells, J. Cell. Physiol. 166:152 169.
Negrotti M, Murray K, Reidy TJ, Krok K, Seaver SS (1989), Effects of glutamine and ammonia on hybridoma cell proliferation and antibody production, Proceedings of Second Engineering Foundation Conference on Animal Cell Culture, Santa Barbara, CA, p. 65 (abstract).
Nelson DL, Cox MM (2004), Lehninger Principles of Biochemistry, 4th Ed., Palgrave MacMillan Publications, Hants, UK.
Newland M, Greenfield PF, Reid S (1990), Hybridoma growth limitations: the roles of energy metabolism and ammonium production, Cytotechnology 3:215 229.
Öhman L, Alarcon M, Ljunggren J, Ramqvist AK, Häggström L (1996), Glutamine is not an essential amino acid for Sf 9 insect cells, Biotechnol. Lett. 18:765 770.
Ozturk SS, Palssom BO (1990), Effects of dissolved oxygen on hybridoma cell growth, metabolism and antibody production kinetics on continuous culture, Biotechnol. Prog. 6:437 446.

Paredes C, Prats E, Cairó JJ, Azorín F, Cornudella L, Gódia F (1999), Modification of glucose and glutamine metabolism in hybridoma cells through metabolic engi neering, Cytotechnology 30:85 93.
Parmeggiani A, Luft JH, Love DS, Krebs EG (1966), Crystallization and properties of rabbit skeletal muscle phosphofructokinase, J. Biol. Chem. 241:4625 4637.
Pessin JE, Bell GI (1992), Mammalian facilitative glucose transporter family. Structure and molecular regulation, Annu. Rev. Physiol. 54:911 930.
Petch D, Butler M (1994), Profile of energy metabolism in a murine hybridoma: glucose and glutamine utilization, J. Cell. Physiol. 161:71 76.
Reuveny S, Velez D, Macmillan JD, Millar L (1986), Comparison of cell propagation methods for their effect on monoclonal antibody yield on fermentors, J. Immunol. Methods 86:61 69.
Rintoul DA, Sklar LA, Simoni RD (1978), Membrane modification of Chinese hamster ovary cells, J. Biol. Chem. 253:7447 7452.
Roberts RS, Hsu HW, Lin KD, Yang TJ (1976), Amino acid metabolism of myeloma cells in culture, J. Cell. Sci. 21:609 615.
Rockwell GA, Sato GH, McClure DB (1980), The growth requirements of SV40 virus transformed Balb/c 3T3 cells in serum free monolayer culture, J. Cell. Physiol. 103:323 331.
Rodriguez J, Spearman M, Huzel N, Butler M (2005), Enhanced production of monomeric interferon β by CHO cells through the control of culture conditions. Biotechnol. Prog. 21:22 30.
Rose DP, Connolly JM (1990), Effects of fatty acids and inhibitors of eicosanoid synthesis on the growth of a human breast cancer cell line in culture, Cancer Res. 50:7139 7144.
Rosenthal MD (1987), Fatty acid metabolism of isolated mammalian cells, Prog. Lipid Res. 26:87 124.
Rotin D, Robinson B, Tannock IF (1986), Influence of hypoxia and an acidic environment on the metabolism and viability of cultured cells: potential implica tions for cell death in tumors, Cancer Res. 46:2821 2126.
Ryll T, Valley U, Wagner R (1994), Biochemistry of growth inhibition by ammonium ions in mammalian cells, Biotechnol. Bioeng. 44:184 193.
Schmid G, Zilg H, Eberhard U, Johannsen R (1991), Effect of free fatty acid and phospholipids on growth of and product formation by recombinant Baby Ham ster Kidney (rBHK) and Chinese Hamster Ovary (rCHO) cells in culture, J. Biotechnol. 17:155 167.
Spector AA, Mathur SN, Kaduce TL, Hyman BT (1981), Lipid nutrition and metabolism of cultured mammalian cells, Prog. Lipid Res. 19:155 186.
Street C, Delort AM, Braddock PSH, Brindle KM (1993), A 1H/15N n.m.r. study of nitrogen metabolism in cultured mammalian cells, Biochem. J. 291:485 492.
Sun XM, Zhang YW (2001a), Effects of glucose on growth, metabolism and EPO expression in recombinant CHO cell cultures, Chin. J. Biotechnol. 17:698 702.
Sun XM, Zhang YX (2001b), Effects of ammonia on cell metabolism in the culture of recombinant CHO cells, Chin. J. Biotechnol. 17:304 309.
Tremblay GB, Mejia NR, MacKenzie RE (1992), The NADP dependent methylenete trahydrofolate dehydrogenase methenyltetrahydrofolate cyclohydrolase formyl tetrahydrofolate synthetase is not expressed in *Spodoptera frugiperda* cells, J. Biol. Chem. 267:8281 8285.
Uyeda K, Racker E (1965), Regulatory mechanisms of carbohydrate metabolim. VII. Hexokinase and phosphofructokinase, J. Biol. Chem. 240:4682 4688.
Valley U, Nimtz M, Conradt HS, Wagner R (1999), Incorporation of ammonium into intracellular UDP activated N acetylhexosamines and into carbohydrate struc tures in glycoproteins, Biotechnol. Bioeng. 64:401 417.
Vriezen N, Romein B, Luyben KCAM, Van Dijken JP (1997), Effects of glutamine

supply on growth and metabolism of mammalian cells in chemostat culture, Biotechnol Bioeng. 54:272 286.
Waddell ID, Zomerschoe AG, Voice MW, Burchell A (1992), Cloning and expression of a hepatic microsomal glucose transport protein, Biochem. J. 286:173 177.
Wagner R (1997), Metabolic control of animal cell culture processes. In: Hauser H, Wagner R (Eds), Mammalian Cell Biotechnology in Protein Production, Walter de Gruyter, Berlin, pp. 193 232.
Wagner R, Ryll T, Krafft H, Lehmannm J (1988), Variation of amino acids concentra tions in the medium of HU β IFN and HU IL 2 producing cell lines, Cytotech nology 1:145 150.
Weidemann R, Ludwig A, Kretzmer G (1994), Low temperature cultivation: a step towards process optimization, Cytotechnology 15:111 116.
Yang M, Butler M (2000), Effects of ammonia on CHO cell growth, erythropoietin production and glycosylation, Biotechnol. Bioeng. 68:370 380.
Yang M, Butler M (2002), Effects of ammonia and glucosamine on the heterogeneity of erythropoietin glycoforms, Biotechnol. Prog. 18:129 138.
Zanghi JA, Schmeltzer AE, Mendoza TP, Knop RH, Miller WM (1999), Bicarbonate concentration and osmolality are key determinants in the inhibition of CHO cell polysialylation under elevated pCO_2 or pH, Biotechnol Bioeng. 65:182 191.

Culture media for animal cells

5

Ângela Maria Moraes, Ronaldo Zucatelli Mendonça, and Claudio Alberto Torres Suazo

5.1 Introduction

Effective *in vitro* maintenance and growth of animal cells requires culture conditions similar to those found *in vivo* with respect to temperature, oxygen and carbon dioxide concentrations, pH, osmolality, and nutrients. Within normal tissue *in vivo*, animal cells receive nutrients through blood circulation. For growth *in vitro*, animal cells require an equivalent supply of a complex combination of nutrients. For this reason, the first attempts in animal cell culture were based on the use of biological fluids such as plasma, lymph and serum, as well as on extracts from embryonic-derived tissue.

Medium composition is one of the most important factors in the culture of animal cells. Its function is to provide appropriate pH and osmolality for cell survival and multiplication, as well as to supply all chemical substances required by the cells that they are unable to synthesize themselves. Some of these substances can be provided by a culture medium consisting of low molecular weight compounds, known as basal media. However, most basal media fail to promote successful cell growth by themselves and require supplementation with more complex and chemically undefined additives such as blood serum.

Many attempts have been made to develop culture media that do not need this type of supplementation, that is, serum-free media formulations. These serum-free media present several practical advantages. However, the formulations that have been developed are specific for certain cell types and a universal culture medium suitable for the culture of all animal cell types seems, so far, unreachable.

While some culture media are formulated to promote cell multiplication (growth media), others only maintain cell structural and metabolic integrity (maintenance media), but do not stimulate cell division. Media prepared with highly purified compounds and with known composition are designated chemically defined media. These are particularly attractive for biopharmaceutical production, because they are less vulnerable to contamination and quality control is easier. Nevertheless, these media can be expensive.

A complete medium for the culture of animal cells can be considered to have two distinct parts. The first supplies basic cell needs of nutrients,

salts, and pH control, while the second comprises a set of supplements that provide other cell needs, allowing cell growth in the basal medium. A nutrient can be defined as a chemical substance that enters into the cell and is used as a structural component, such as a substrate for cell biosynthesis or energy metabolism, or as a catalyst in metabolic processes. Additional compounds required for cell proliferation can be considered as supplements, including all undefined additives such as serum and other biological fluids.

Media frequently employed in the culture of continuous mammalian cell lines include: Eagle's medium, MEM (Eagle, 1959); Eagle's medium modified by Dulbecco, DMEM (Dulbecco and Freeman, 1959); RPMI 1640 medium (Moore et al., 1967); CMRL™ 1066 medium (Parker *et al*., 1957); and Ham's F12 medium (Ham, 1965). For the cultivation of adherent continuous cell lines the following basal media are suitable: CMRL™ 1066, MCDB 411, DMEM. F12, MCDB 301, and IMDM. For non-transformed cells, the media DMEM, IMDM, MCDB 104, 105, 202, 401, and 501 are suitable (Freshney, 1992). Each of these basal formulations may be supplemented with serum or other specific proteins.

For insect cells the following basal media can be used: Grace's, TC 100, TNM-FH, D22, Schneider, and M3. These media normally require supplementation with fetal bovine serum. Alternatively, different serum-free media are available for insect cells, such as Sf900II, Ex-Cell® 400, 405, and 420, Express Five® SFM, Insect-XPRESS™, HyQ SFX-Insect™, and IPL 41. These have the advantage of higher reproducibility and lower cost when compared with serum-supplemented basal media (Ikonomou *et al.*, 2003).

For comparison purposes, *Table 5.1* shows the composition of DMEM (one of the most versatile medium formulations, typically employed in the culture of mammalian cells) and Schneider's medium (used for diptera insect cells, especially *Drosophila melanogaster*). A total of 34 different components are present in DMEM, although some minor variations in composition may occur between suppliers.

Table 5.1 Composition of minimum Eagles's medium modified by Dulbecco, DMEM, used for mammalian cells, and of Schneider's medium, used in the culture of dipteran cells

Component	Concentration in medium (mg/ml)	
	DMEM	Schneider
Amino acids		
L Alanine		500,0
Glycine	30.0	250.0
L Aspartic acid		400.0
L Glutamic acid		800.0
L Arginine		600.0
L Arginine hydrochloride	84.0	
L Cysteine		60.0
L Cystine	48.0	

Table 5.1 (continued)

Component	Concentration in medium (mg/ml)	
	DMEM	Schneider
L Cystine dihydrochloride		26.7
L Glutamine	584.0	1800.0
L Histidine		400.0
L Histidine hydrochloride.H_2O	42.0	
L Isoleucine	105.0	150.0
L Leucine	105.0	150.0
L Lysine hydrochloride	146.0	2060.8
L Methionine	30.0	150.0
L Phenylalanine	66.0	
L Proline		1700.0
L Serine	42.0	250.0
L Threonine	95.0	350.0
L Tryptophan	16.0	100.0
L Tyrosine	72.0	
L Tyrosine 2Na		72.0
L Valine	94.0	300.0
Vitamins		
Choline chloride	4.0	
D Pantothenate Ca	4.0	
Folic acid	4.0	
i Inositol	7.2	
Nicotinamide	4.0	
Pyridoxine hydrochloride	4.0	
Riboflavin	0.4	
Thiamine hydrochloride	4.0	
Inorganic salts		
Calcium chloride ($CaCl_2$)	200.0	600.0
Ferric nitrate ($Fe(NO_3)_3.9H_2O$)	0.1	
Magnesium sulfate heptahydrate ($MgSO_4.7H_20$)	200.0	
Magnesium sulfate		1810.0
Potassium chloride (KCl)	400.0	1600.0
Monobasic potassium phosphate (KH_2PO_4)		450.0
Sodium bicarbonate ($NaHCO_3$)	3700.0	400.0
Sodium chloride (NaCl)	6400.0	2100.0
Monobasic sodium phosphate monohydrate ($NaH_2PO_4.H_2O$)	125.0	
Monobasic sodium phosphate (NaH_2PO_4)		700.0
Other components		
D Glucose (dextrose)	4500.0	2000.0
D Threalose		2000.0
Phenol red	15.0	
Sodium pyruvate	110.0	
Linoleic acid	0.084	
Fumaric acid		60.0
α Ketoglutaric acid		350.0
Malic acid		600.0
Succinic acid		60.0
Yeast extract		2000.0

5.2 Main components of animal cell culture media

The culture medium must contain nutrients essential to the synthesis of new cells and substrates for accomplishing cell metabolism, besides compounds allowing physiological and catalytic functions, or which act as cofactors. To maintain the conditions found in the original tissue from which a particular cell originated, it is necessary for a culture medium to contain: inorganic salts, sugars, amino acids, vitamins, lipids, organic acids, proteins, hormones, carbon and nitrogen sources, micronutrients (organic ions and minerals), and water as well as cell-specific substances. Sometimes, blood serum and antibiotics are also required, and in addition, the culture must be kept free from inhibiting or toxic compounds.

Information on animal cell metabolism is essential for the formulation of new culture media, as well as for the composition of the most appropriate nutrient feed that may be used in various strategies of batch, semi-batch or continuous culture that may be designed for the production of the targeted protein. As already discussed in detail in Chapter 4 and concisely presented in *Figure 5.1*, two fundamental substrates for animal cells are glucose and glutamine.

The following sections will outline the major components of a typical culture medium for animal cells as well as their typical concentrations and their functions in cell metabolism.

5.2.1. Water

One of the basic components of a culture medium, and also one of the most critical, is water. Animal cells are extremely sensitive to water

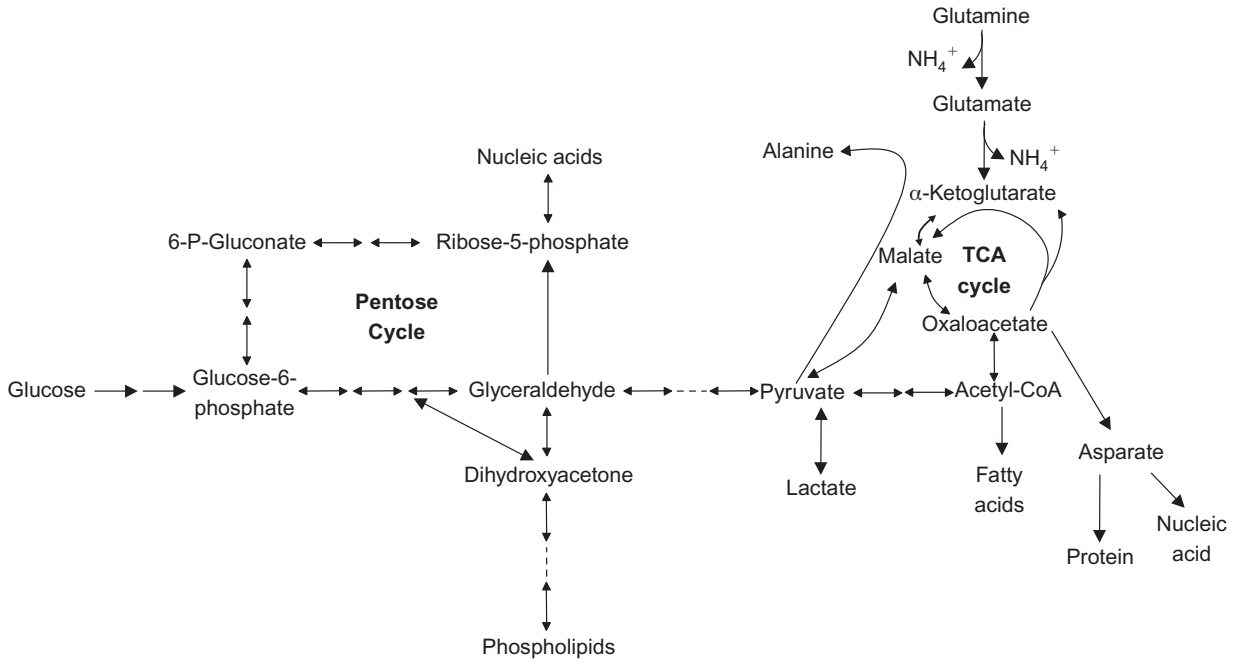


Figure 5.1

Major pathways for central metabolism in animal cells (based on Shuler and Kargi, 2002).

quality, since this can be the source of contamination that can affect cell growth. Potential contaminants include inorganic compounds (heavy metals, iron, calcium, and chloride), organic compounds (detergents), microbial-related contaminants (endotoxins and pyrogens), besides particles and colloids from several different origins. Since the presence of any of these contaminants can prevent cell culture on any scale, strict purity standards of water quality must be maintained. Efficient water purification systems are, therefore, required, and can be based on multiple-distillation systems or on equipment combining deionization, microfiltration, and reverse osmosis. The water used in the preparation of industrial culture media should be subjected to similar purification methods and should be continuously monitored to ensure the physicochemical and microbiological standards established by national pharmacopoeia. The standards for water employed for pharmaceutical use in different countries are described in *Table 5.2*, both for water for injection (WFI) and for purified water (PW). While PW is used for the preparation of products not requiring sterile or apyrogenic water, WFI meets the standards for PW as well as for bacterial endotoxins, conductivity, and total organic carbon.

5.2.2 Glucose

Glucose is usually the main carbohydrate for animal cell growth, acting as source of both carbon and energy (Bérdad *et al.,* 1993; Drews *et al*., 1995; Öhman *et al.,* 1995; Mendonça *et al.,* 1999; Freshney, 2005). Normally, this compound is added to the culture medium in concentrations varying from 5 to 25 mM (0.9–4.5 g/L), but may be up to 56 mM (10 g/l).

Table 5.2. Standards for the quality of purified water (PW) and water for injection (WFI) (Slabicky, 1994)

Parameter	Units	USA		Great Britain		Europe		Japan	
		PW	WFI	PW	WFI	PW	WFI	PW	WFI
Acidity/alkalinity	$mg.L^{-1}$			1	2	1	2	0.1	0.1
Ammonium	$mg.L^{-1}$	0.3	0.3	0.2	0.2	0.2	0.2	0.05	0.1
Bacterial endotoxin	$EU.L^{-1}$		0.25		Pyrogen		Pyrogen		0.25
Calcium	$mg.L^{-1}$	0.5	0.5	1	1	1	1		
Carbon dioxide	$mg.L^{-1}$	4	4						
Chlorides	$mg.L^{-1}$	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Heavy metals (Pb)	$mg.L^{-1}$	0.5	0.5	1	1	0.1	0.1	1	
Magnesium	$mg.L^{-1}$			0.6	0.6	0.6	0.6		
Nitrates (N)	$mg.L^{-1}$			0.2	0.2	0.2	0.2	0.2	0.2
Oxidizable substances	$mg.L^{-1}$	5	5	1	0.5	5	10	5	
pH		5 7	5 7						
Sulfates	$mg.L^{-1}$	0.5	0.5	0.5	0.5	0.5	0.5	0.5	
Total organic carbon	$mg.L^{-1}$								0.500
Total solids	%	0.001	0.001	0.001	0.003	0.001	0.003	0.001	0.003

PW, purified water; WFI, water for injection; EU, endotoxin units; Pyrogen, pyrogen test (qualitative).

As detailed in Chapter 4, glucose is metabolized mainly through glycolysis, forming pyruvate, which can be converted to lactate or acetyl that can enter into the citric acid cycle, forming carbon dioxide and water. Lactate accumulation in the culture medium indicates that the citric acid cycle may not function *in vitro* similarly to the way it does *in vivo*. There is evidence that a significant amount of carbon may be assimilated from glutamine, and not from glucose, and that explains the large requirement for this amino acid by some cell types. Insect cells, in general, accumulate lactate at lower levels than mammalian cells. However, lactate can still be produced by these cells in media with low oxygen concentrations.

The accumulation of lactate in high density cultures is a problem, since it causes medium acidification. Also, this compound is toxic to the cells, inhibiting cell growth when present in large concentrations. The substitution of part of the glucose by mannose, fructose, galactose, or maltose reduces glucose consumption rate and, as a consequence, also reduces the lactate formation rate. However, total substitution may affect protein glycosylation patterns, altering proteins such as antibodies.

5.2.3 Amino acids

Essential amino acids are those not synthesized by the organism and are required by animal cells in culture. These include specific amino acids such as cysteine and tyrosine but the requirement varies between cell lines. Amino acids are necessary for protein, nucleotide, and lipid synthesis and, in addition, may be used as an energy source.

These compounds can be provided as a defined mixture or in the form of protein hydrolysates, such as lactoalbumin, or plant-derived hydrolysates, like colza (Deparis *et al*., 2003), soybean (Donaldson and Shuler, 1998; Heidemann *et al*., 2000; Ikonomou *et al.*, 2001), wheat (Heidemann *et al*., 2000; Ikonomou *et al.*, 2001; Ballez *et al.*, 2004), and rice (Heidemann *et al.*, 2000; Ikonomou *et al.*, 2001; Ballez *et al.*, 2004). Supplementation with yeast extract also provides additional amino acids.

Traditionally, from 0.1 to 1 mM of each amino acid is added to the culture medium, including both essential and non-essential amino acids. The concentrations of amino acids added to insect cell culture media are much higher than those found in media for vertebrate cells (Echalier, 1997), probably due to the fact that higher amino acid concentrations are found in insect hemolymph (insect body fluid) in comparison with blood serum.

Glutamine, methionine, and serine are growth-limiting amino acids (Freshney, 1992). Glutamine acts as a source of carbon, nitrogen, and energy, and is normally added to the culture medium at high concentrations, varying from 1 to 5 mM. Special attention should be given to its stability, since glutamine spontaneously degrades by cyclization to pyrrolidone carboxylic acid in the culture medium. Glutamine is prone to thermal degradation (at 35°C, for instance, glutamine is 50% degraded in only 9 days), and therefore, should be periodically replenished when required. The main metabolite generated from glutamine metabolism is ammonium which, similarly to lactate, presents toxic effects and is a cell growth inhibitor when accumulated in large quantities. It can be converted

enzymatically to glutamic acid, leucine, and isoleucine by enzymes found in cells and also in the blood serum normally added to culture media (Freshney, 1992).

5.2.4 Vitamins

Many cells require media supplemented with complex B vitamins, while other vitamins are presumably supplied by the addition of serum to culture media. Nevertheless, when serum-free media are employed, not only the water-soluble vitamins should be provided, but also the lipid-soluble ones, such as biotin, folic acid, niacin, panthotenic acid, thiamine, and ascorbic acid, as well as the vitamins B_{12}, A, D, E, and K.

Vitamins are used in very low amounts as enzyme cofactors, essential for general cell metabolism. While ascorbic acid acts in collagen synthesis, vitamin A affects cell growth and differentiation. Vitamin K is required for gamma-carboxylation and for correct protein processing. Vitamin E is an antioxidant agent, whereas vitamin D regulates calcium ion transport and acts as a hormone, being toxic when supplied in excess.

Besides serum, yeast extract can also be used as an effective vitamin supply to culture media.

5.2.5 Salts

The salts most commonly added to the culture medium are Na^+, K^+, Mg^{2+}, Ca^{2+}, Cl^-, SO_4^{2-}, PO_4^{3-}, and HCO_3^-. These ions are important in the maintenance of the ionic balance and osmotic pressure, besides acting as enzymatic cofactors. Salts are the components that contribute most to the increase in culture medium osmolality. Na^+, K^+, and Cl^- have relevant action in the regulation of the cellular membrane potential, while SO_4^{2-}, PO_4^{3-}, and HCO_3^- are important for macromolecular synthesis, as well as regulators of intracellular charge (Freshney, 2005). In addition, HCO_3^- acts in the binding of iron to transferrin and in culture medium buffering.

For suspension cultures, the concentration of calcium and magnesium should be kept low to prevent cell aggregation and adhesion. Other metals, such as iron, manganese, selenium, vanadium, zinc, copper, and molybdenum, are usually added to the culture medium, but at reduced concentrations, and mainly if the medium is not supplemented with animal serum.

5.2.6 Serum

Blood serum, usually bovine-derived (calf or fetal bovine), contains amino acids, growth factors, vitamins, proteins, hormones, lipids, and minerals, among other components, as indicated in *Table 5.3*. Besides fetal bovine serum, serum from horse (equine), and even from humans (less common) can also be used. The main functions of serum are to stimulate growth and other cellular activities through hormones and growth factors, to increase cellular adhesion through specific proteins, and to supply proteins for the transport of hormones, minerals, and lipids (Freshney, 2005). Supplementation with bovine fetal serum is performed at concentrations from 2 to 20% in volume.

In the case of insect cells, insect hemolymph can be used as an alternative to bovine serum. However, the availability of hemolymph is much more restricted.

5.2.7 Other components necessary for cell culture

Besides the compounds already mentioned, some adherent cell lines need proteins of the extracellular matrix (ECM) for efficient adherence to the support and for cell growth. Many ECM proteins, such as fibronectin, are present in serum, as shown in *Table 5.3*, while others, such as collagen, are secreted by cells constitutively or after their stimulation with a growth factor. Another ECM protein, laminin, can

Table 5.3 Serum components and their functions in the animal cells cultured *in vitro* (adapted from Freshney, 2005)

Component	Probable function
Proteins	
Albumin	Osmotic control and buffering agent, transport of lipids, hormones and minerals, mechanical protection
Fetuin	Cell attachment
Fibronectin	Cell attachment
α_2 Macroglobulin	Trypsin inhibitor
α_1 Antitrypsin	Trypsin inhibitor
Transferrin	Binds iron
Growth factors	
Endothelial growth factor (ECGF)	Mitogen
Epidermal growth factor (EGF)	Mitogen
Fibroblast growth factor (FGF)	Mitogen
Insulin like growth factors (IGF 1 and IGF 2)	Mitogen
Platelet derived growth factor (PDGF)	Mitogen and major growth factor
Interleukin 1 (IL 1)	Induces interleukin 2 release
Interleukin 6 (IL 6)	Promotes differentiation
Hormones	
Hydrocortisone	Promotes attachment and proliferation
Insulin	Promotes glucose and amino acids uptake
Growth hormones	Mitogen (present in fetal serum)
Metabolites and nutrients	
Amino acids	Cellular proliferation
Glucose	Cellular proliferation
Keto acids (e.g. pyruvate)	Cellular proliferation
Lipids (e.g. cholesterol)	Membrane synthesis
Minerals	
Iron, copper, zinc, selenium	Enzyme cofactors and constituents
Inhibitors	
γ Globulin	
Bacterial toxins from prior contaminants	
Chalones (tissue specific inhibitors)	

be used as an alternative to fibronectin, especially for epithelial cell cultures. Growth factors, present in very low amounts, are usually small peptides (5–30 kDa). Among them, the following types are prominent: FGF (fibroblast growth factor), EGF (epidermal growth factor), NGF (nerve growth factor), TGF (transforming growth factor), PDGF (platelet-derived growth factor), the insulin homologous IGF-1 and IGF-2, and interleukins (Jenkins, 1991).

According to Jenkins (1991), the mitogenic factor FGF is isolated from bovine brain in two forms, acid FGF (pI 5.6) and basic FGF (pI $>$ 9.0). These factors bind strongly to heparin. Basic FGF (bFGF) presents mitogenic action through calcium influx and activation of quinase C proteins, being a potent mitogenic agent for several types of cultures derived from mesodermal and neuroectodermal cells, as well as for several transformed cell lines. This compound also promotes differentiation of adipocytes and ovary granulous cells, inducing the synthesis of ECM proteins such as collagen type IV. bFGF has been used to enhance the survival of new cell lines derived from primary cultures and also to eliminate the use of feeder cells during hybridoma cloning. Feeder cells (such as irradiated fibroblasts or cells from peritoneal exudates) are used to condition culture media with an undefined mixture of growth-promoting substances, particularly when hybridomas are present at low densities.

EGF can be isolated from mouse submaxillary glands and also from human urine. It is a potent mitogenic agent for many primary cultures and for mesenchymal and epithelial cells, having a synergistic effect with other growth factors, such as IGF-1 and TGFβ.

NGF is another factor purified from mouse submaxillary glands, and it can also be obtained from bovine brain and placenta. NGF is not a potent mitogenic agent, but it induces differentiation and increases the survival of sympathetic neurons and PC12 cells in culture.

The factors TGFα and TGFβ were initially observed in fibroblast cultures of mouse kidney cells transformed by retrovirus (Sporn *et al*., 1987). TGFα induces cellular growth independently of adhesion, and its action is similar to that of EGF (Derynck, 1988). However, several cell lines that produce endogenous TGFα, like A431, can have their growth inhibited by EGF. TGFα is found naturally in embryonic kidney, adult brain, pituitary gland, skin, and in the placenta.

TGFβ is a factor commonly purified from human platelets, which predominantly secrete TGF-1 (Jenkins, 1991). The biologically inactive TGF precursor (220–235 kDa) circulates in the blood as a protein and possible protease carrier. Most of the mammalian cells in culture secrete small amounts of TGFβ precursor and have TGFβ receptors. TGFβ promotes growth in several lineages of mesenchymal cells when in combination with TGFα or EGF. In contrast, isolated TGFβ inhibits the growth of many cell lines in monolayer culture, which could result from its capacity to stimulate the secretion of ECM proteins (collagen, fibronectin, and glucosaminoglycans) and protease inhibitors (Moses *et al*., 1987). The inhibition of monolayer growth by TGF is more prominent in epithelial cells, endothelial cells (antagonized by the bFGF), stem cells, and lymphocytes.

PDGF is one of the most common serum components (40–60 ng/ml). It is also secreted by endothelial and placenta cells, and by several cell lines such as BHK and 3T3-fibroblasts. PDGF has synergetic action with EGF and IGF-1 and is a potent mitogen.

Another fundamental compound for cellular growth is insulin, which presents weak affinity to IGF-1 receptors. Insulin can activate several mitogenic responses, through IGF-1 receptors when in large doses. However, insulin is also added to most serum-free media due to its ability to promote anabolic metabolism, such as oxidation processes, glycogen synthesis, and amino acids transport (Jenkins, 1991).

Peptides homologous to insulin can also act as growth factors, such as somatomedin-C (Daughaday and Rotwein, 1989). IGF-1, secreted in small amounts by many tissues, acts on several mesenchymal cells, while IGF-2, produced by fetal liver, muscle, skin, and adult brain, is known for its growth stimulatory activity.

Some cells, such as hybridomas, stem, and hematopoietic cells may require a class of proteins known as interleukins, especially IL-6. This compound can substitute for feeder cells (such as those of the peritoneal exudates or spleen, fibroblasts or timocytes) in the post-fusion stage of hybridoma cultures. IL-6 is secreted by monocytes, T lymphocytes, and endothelial cells and is effective in the stimulation of hybridomas of different species, including lineages that are difficult to cultivate, such as human–mouse and rat–mouse hybrids. IL-6 also presents a synergistic effect with other interleukins to increase antibody production.

Some growth factors act as carrier proteins in the culture medium. These proteins can serve as carriers in lipid transport (as in the case of the albumin) or for metal ion transfer to cells (such as transferrin). Albumin can act as a buffering or a detoxifying agent (due to its binding to endotoxin and pyrogens), as well as serving as a carrier for vitamins, hormones, or other micronutrients. Transferrin, on the other hand, is a protein with a large capacity as an iron carrier. Many mammalian cells possess transferrin-specific receptors which capture transferrin/Fe^{3+} complexes.

Lipids also can be beneficial for cells in culture, since some substances absorbed by the cells need to be solubilized in lipids, or in some cases the toxicity of compounds may be reduced by complexation with lipids. The absence of essential lipids such as linoleic acid, lecithin, cholesterol, ethanolamine, or phosphorylcholine can result in the decrease of cloning efficiency and in reduction in the size of colonies, as shown for insect cells by Echalier (1997). However, one of the difficulties in supplying lipids at reasonable concentrations is their low solubility. To circumvent this limitation, lipids can be emulsified with complexing agents such as Pluronic® F68 or cyclodextrin (Maiorella *et al*., 1998).

Antibiotics such as penicillin, streptomycin, and amphotericin B can also be added to the culture medium to prevent microbial growth. However, their use should be kept to a minimum level, because some antibiotics can have cytotoxic effect, even when high serum concentrations are used. Besides, the continuous use of antibiotics can result in the selection of resistant microorganisms, inducing a chronic contamination of the culture (Freshney, 1992).

5.3 Advantages and limitations of the use of media supplemented with animal serum

As already mentioned, the most frequently employed serum in animal cell culture is bovine fetal serum; however, sera of calves, horses, and even human sera are also used. Proteins of the human plasma are obtained in substantial amounts as a result of routine therapeutic production of blood products. In these conditions, large amounts of albumin, transferrin and insulin, as well as growth factors, can be obtained. The fractions with no therapeutic application are usually discarded, but they can be processed with the purpose of supplementing low protein media, as a direct substitute for bovine fetal serum, allowing the growth of a wide range of cells. To use these proteins, cell adaptation to the medium containing the new products is necessary, and this process can take several weeks. In addition, limitations such as the possibility of introduction of human pathogens in the culture should be considered.

Fetal bovine serum is the favorite because it presents low immunoglobulin and high growth factor concentrations. Its main advantage is that it allows the same medium to culture different cell lines. For production, the blood of bovine fetuses is collected by heart puncture and, after slow coagulation, it is centrifuged, resulting in the serum in the supernatant. The entire process should be performed at low temperatures, and the animals should be free from infectious diseases, such as rinderpest, foot-and-mouth (aftosa), and bovine spongiform encephalopathy (''mad cow disease''). Fetal bovine serum is scarce since, on average only 7–9% of slaughtered animals are estimated to be pregnant and each fetus supplies from 0.2 to 0.5 l of blood with only half the volume becoming serum. Thus, around 44–144 animals have to be slaughtered to obtain 1 l of fetal bovine serum. Beside the low process yield, there is also an ethical aspect. The animals can suffer if at the moment of blood collection their lungs are insufflated with air, increasing the oxygen blood concentration to levels compatible with animal consciousness (Van der Valk *et al*., 2004).

In addition to the factors that increase cellular adhesion and the nutritional factors, serum also presents culture-stimulating (growth factors, hormones and proteins) and protecting agents, both for biological protection (antitoxin, antioxidant, antiprotease) and for prevention of mechanical damage. However, fetal serum is one of the most expensive components in culture medium. In addition, serum supplementation can significantly increase the complexity and the cost of the targeted product recovery and purification process due to the presence of many proteins and growth factors, among other components, most of them undefined. Bovine fetal serum can contain more than 1000 different components.

Another factor to be taken into account when using animal serum is the potential risk for human health, due to the possible presence of adventitious agents, such as virus and proteins as prions. In addition, serum can contain contaminants such as bacteria, fungi, and mycoplasmas (small bacteria without cellular walls), which can negatively affect cell culture.

Another strong limitation to the use of bovine fetal serum is its variability between different lots and suppliers, which hinders the standardization of the culture medium and the reproducibility of culture perform-

ance. Since several animal cell lines can grow in serum-free media, or in media supplemented with certain components found in bovine fetal serum, as part of good practice for cell cultivation, the use of bovine fetal serum should be avoided (Van Valk *et al*., 2004).

Within the last few years there has been a strong recommendation from regulatory agencies such as the FDA (Food and Drug Administration, USA), not to use animal-derived components in media formulation for the production of biopharmaceuticals and other compounds destined for therapeutic application in humans.

5.4 Strategies to formulate serum-free culture media

The first culture medium with a defined composition was developed in the 1950s. In 1959, Eagle developed the ''minimal essential medium'' for mammalian cell culture, composed of glucose, amino acids, vitamins, and a solution of balanced salts. In 1962 Grace accomplished the same with a culture medium for insect cells (Grace, 1962). Within the last 50 years there has been considerable work in the search for more efficient culture media or to meet the specific needs of some cell lines. Such media were always based on minimal formulations supplemented with fetal serum, which as previously discussed, acts as a source of enzymes, hormones, and microelements necessary for cell growth. However, since adding serum has many disadvantages, the development of serum-free media with a defined composition has intensified in recent years. Formulating medium for animal cells requires an evaluation of the effect of each constituent so that the optimal composition is attained.

Any new formulation may be evaluated against the properties of a serum-substituted medium for growth and productivity. As well as growth promotion, a serum supplement in medium may also lead to a reduction in cell death by apoptosis and this allows extended culture longevity. This can enable a substantial increase in recombinant protein productivity.

In a multi-component system such as the cell environment, many factors are interconnected, and a specific compound may be essential in some but not all situations. Although an initial medium formulation is often based on the composition of the circulating fluid of the organism, some nutrients essential for metabolic activity in tissues and organs may be completely unnecessary for *in vitro* cell proliferation.

One approach to developing a medium with a defined composition is to identify the existing elements in fetal serum that have a positive effect in cell culture. Serum may then be substituted by adding specific components with similar functions, based on the effect of each supplement on cell growth (Barnes and Sato, 1979, 1980; Barnes *et al*., 1984a, 1984b, 1984c, 1984d; Taub and Livingston, 1981). To determine the importance of a specific component, cultures can be monitored with or without its presence at quantities normally found under physiological conditions. Afterwards, the concentrations can be varied and the effect of each specific component can be optimized.

The key strategy when a new medium is developed is to reproduce the metabolic conditions of the cells when *in vivo*. Therefore, when adding media components it is important to maintain the pH, redox potential, and osmolality constant, as well as attempting to avoid generation of metabolic compounds that are toxic or growth inhibitory, such as lactate and/or ammonium. Apart from purely nutritional factors, it is important to develop a medium that is stable under normal storage conditions or in contact with light.

Currently, due to the wide use of cell culture in the biopharmaceutical, therapeutic, and diagnostic areas, the development of new media has been critical, in an attempt to minimize the number of components, maximizing efficiency, or simplifying the purification processes. Thus, media with many characteristics and distinct functions have been developed.

In the development of an alternative culture medium for a given cell line, it must be ensured that the components not being studied are provided in sufficient quantity, such as oxygen, which can behave as a limiting substrate in some situations. The assessment of cellular performance in different culture media is usually challenging and time-consuming, since it requires establishment of the concentration of total and viable cells, over multiple passages. In addition it may involve the quantification of residual nutrients, such as glucose and glutamine, of metabolic byproducts, such as lactate, ammonium and alanine, and the concentration of the target product. Equally relevant is the use of cellular inoculum of appropriate age and concentration. One of the greatest difficulties is to design a culture medium that ensures replicate cultures that show stable genetic, metabolic, and kinetic behavior.

Among the strategies used to establish new formulations are: univariate analysis (where the effect of only one component is studied at a time), analysis of the effect of a group of nutrients, and statistical design of experiments.

The strategy of statistical design of experiments (Barros Neto *et al.*, 1995; Rodrigues and Iemma, 2005) appears to be a rational and efficient way for a direct and quick determination of the effect and interaction among the parameters analyzed (Montgomery and Runger, 1999). This type of procedure results in the minimization of cost and time. This strategy has proved to be adequate to qualitatively analyze the effects of the use of protein hydrolysates as supplements for insect cell cultivation (Ikonomou *et al.*, 2001; Batista *et al.,* 2005).

The new media that are commercially available possess many distinct characteristics; some are completely free of animal-derived components, others are serum-free, sometimes requiring addition of some protein fraction or containing a discrete quantity of proteins. Another class is protein-free media, which can include components derived from animals, plants, or yeast hydrolysates. In some cases, the development of novel media is unnecessary, as it is sufficient to supplement basal medium with a few known specific components. It is emphasized that the final osmolality of the formulated medium should be compatible with cellular tolerance, as discussed in Chapter 2.

Protein hydrolysates may provide an alternative to a serum supplement. Such hydrolysates contain oligopeptides, peptides, and amino acids ob-

tained by chemical or enzymatic hydrolysis of caseins, plants, animal tissues, or yeast (Ikonomou *et al.,* 2003). Some of the most commonly used hydrolysates are bacto-peptone (Keay, 1976), proteose peptone (Hasegawa *et al.,* 1988), and Primatone® RL (Mizrahi, 1977; Schlaeger *et al*., 1993; Schlaeger, 1996). Primatone® RL, a commercially available meat digest, is a low-cost supplement that serves as a source of amino acids, oligopeptides, iron salts, lipids, and traces of other low molar mass substances.

Recently, plant hydrolysates have also been tested as additives for serum-free culture media (Donaldson and Shuler, 1998; Franek *et al.,* 2000; Heidemann *et al.,* 2000). Donaldson and Shuler (1998) demonstrated that a peptide obtained from soy hydrolysate (Hy-Soy®) played an important role in a serum-free medium developed for insect cells. Ikonomou *et al.* (2003) also showed the effectiveness of culture medium supplemented with the soy hydrolysate SE50MAF in the growth of High Five™ cells.

Further than providing nutrients, these hydrolysates may play an essential role in providing growth factors, antiapoptotic factors, or protein production stimulators in serum-free cultures (Ikonomou *et al.*, 2003).

As well as these hydrolysates, other supplements can also totally or partially substitute serum, such as yeast extract (Drews *et al*., 1995; Wu and Lee, 1998; Wu *et al*., 1998; Ikonomou *et al*., 2001, 2003; Sung *et al*., 2004; Batista *et al.*, 2005, Mendonça *et al*., 2007) and peptides obtained from insect hemolymph (Maranga *et al*., 2003; Souza *et al*., 2005). While yeast extract is mainly used as a source of vitamins (Mitsuhashi, 1989), stimulating cell growth, the peptides obtained from insect hemolymph have a potent effect in inhibiting apoptosis and positive activity in cell growth and in producing recombinant proteins (Maranga *et al.*, 2003).

Some cell lines, such as Namalwa, can grow satisfactorily in medium in which the only protein is albumin. Other cell lines show distinct protein requirements, such as albumin, transferrin, and insulin, or the addition of polypeptide growth factors, isolated from non-serum sources that have shown stimulation of many cell types in culture. Some cells have very fastidious growth requirements and their stability and productivity may be reduced significantly in serum-free media.

The chemically defined media, that is, those that may contain a specific protein or a specific animal extract compound, as long as they are purified and have known concentration of all components, are ideal to eliminate production variability, increasing reproducibility, facilitating and reducing the costs of protein purification, and eliminating the potential risk of pathogenic agent transmission. Many components of animal origin that are necessary for cell growth can be substituted by bioactive synthetic chemical products or by compounds originating from microbial fermentation. The formulation of chemically defined media can allow more in-depth studies, especially metabolic and regulation studies, as well as the fact that their use can facilitate approval of products derived from animal cells by the regulatory agencies.

Despite all the development seen, up to the present time there is no standard culture medium that could be universally utilized for all cells, since each cell line has a specific metabolic profile and requires an

appropriate medium composition. The most difficult part of the medium development process is to predict exactly which supplement or set of supplements will be necessary for each cell line. Nevertheless, useful information can be obtained from work on cell lines of the same origin. Recent advances in cellular biology and molecular genetics have allowed the development of new cell lines and this has intensified the demand for new culture media.

Generally, in terms of the growth factor requirements, cells can be classified in four categories, as discussed below (Jenkins, 1991). In the first category are the transformed cell lineages, such as CHO, BHK, melanoma, hybridoma, and myeloma cells, which go through the cell cycle, only needing some maintenance factors. Normally, these cells need insulin (5 μg/ml), serum albumin, lipid- and vitamin-carrying proteins, transferrin (5 μg/ml for iron transport), and microelements (Jenkins, 1991).

The second category includes mesenchymal cells, such as fibroblasts (BALB/c 3T3, Swiss 3T3), adipocytes, endothelial cells, smooth thin muscle cells, and neuroectodermic cells (such as glia cells). Most of these cells need maintenance factors. Some cells, such as the NIH-3T3, can grow in a serum-free medium containing minimal medium supplemented with transferrin (25 μg/ml), insulin (10 μg/ml), EGF (100 ng/ml), bFGF (100 ng/ml), and PDGF (0.5 U/ml).

The cells from the third category, such as epithelial cells isolated from mammal glands, testes, ovaries, pituitary gland, prostate, and from ocular tissue, generally proliferate with more difficulty in a defined medium. Besides maintenance factors, these cells may also need proteins from the ECM to promote their adhesion. Those proteins can be collagen, fibronectin, or laminin. Non-peptide factors, such as retinol (50 ng/ml) and corticosteroids (10–100 ng/ml), may also be necessary.

Finally, in the fourth and last category are certain types of cells (PC12, hybridomas, and hepatic cells) that may also need factors such as NGF, IL-6, and growth hormones.

When preparing a new medium the need for cell adaptation should be considered. The adjustment process for low protein media is often carried out in static cultures employing tissue culture flasks. However, problems seen at the time of adaptation can be minimized in suspension cultures, since this allows subculture during the log growth phase. Cultures in suspension also allow better medium oxygenation. However, under any condition and with any medium, there will always be a need for some cell adaptation to the new medium to stabilize cellular replication. As soon as a cell population is adapted for growth in a low protein medium, it is recommended that a cell bank is established.

References

Ballez JS, Mols J, Burteau CC, Agathos SN, Schneider YJ (2004), Plant protein hydrolysates support CHO 320 cells proliferation and recombinant interferon gamma production in suspension and inside microcarriers in protein free media, Cytotechnology 44:103 114.

Barnes D, Sato G (1979), Growth of a mammary tumor cell line in a serum free medium, Nature 281:388 389.

Barnes D, Sato G (1980), Methods for growth of cultured cells in serum free medium, Anal. Biochem. 102:255 270.
Barnes DW, Sirbasku DA, Sato GH (1984a), Methods for Preparation of Media, Supplements, and Substrata for Serum free Animal Cell Culture, Alan R Liss, New York, p. 355.
Barnes DW, Sirbasku DA, Sato GH (1984b), Methods for Serum free Culture of Cells of the Endocrine Systems, Alan R Liss, New York, p. 255.
Barnes DW, Sirbasku DA, Sato GH (1984c), Methods for Serum free Culture of Neuronal and Lymphoid Cells, Alan R Liss, New York, p. 263.
Barnes DW, Sirbasku DA, Sato GH (1984d), Methods for Serum free Culture of Epithelial and Fibroblastic Cells, Alan R Liss, New York, p. 291.
Barros Neto B, Scarminio IS, Bruns RE (1995), Planejamento e Otimização de Experimentos, 2nd Ed., Editora da UNICAMP.
Batista FRX, Pereira CA, Mendonça RZ, Moraes AM (2005), Enhancement of Sf9 cells and baculovirus production employing Grace's medium supplemented with milk whey ultrafiltrate, Cytotechnology 49:1 9.
Bérdad C, Tom R, Kamem A (1993), Growth, nutrient consumption, and end product accumulation in Sf9 and BTI EAA insect cell cultures: insights into growth limitation and metabolism, Biotechnol. Prog. 9:615 624.
Daughaday WH, Rotwein P (1989), Insulin like growth factors I and II. Peptide, messenger ribonucleic acid and gene structures, serum, and tissue concentrations, Endocr. Rev. 10:68 91.
Deparis V, Durrieu C, Schweizer M, Marc I, Goergen Jl, Chevalot I, Marc A (2003), Promoting effect of rapeseed proteins and peptides on Sf9 insect cell growth, Cytotechnology 42:75 85.
Derynck R (1988), Transforming growth factor alpha, Cell 54:593 595.
Donaldson MS, Shuler ML (1998), Low cost serum free medium for the BTI Tn5B1 4 insect cell line, Biotechnol. Prog. 14:573 579.
Drews M, Paalme T, Vilu R (1995), The growth and nutrient utilization of the insect cell line Spodoptera frugiperda Sf9 in batch and continuous culture, J. Biotechnol. 40:187 198.
Dulbecco R, Freeman G (1959), Plaque production by the polyoma virus, Virology 8:396 397.
Eagle H (1959), Amino acid metabolism in mammalian cell cultures, Science 130: 432 437.
Echalier G (1997), *Drosophila* cells in Culture, Academic Press, New York.
Franek F, Hohenwarter O, Katinger H (2000), Plant protein hydrolysates: preparation of defined peptide fractions promoting growth and production in animal cell cultures, Biotechnol. Prog. 16:688 692.
Freshney RI (1992), Animal Cell Culture A Practical Approach, 2nd Ed., IRL Press, Oxford.
Freshney RI (2005), Culture of Animal Cells. A Manual of Basic Technique, 5th.Ed., John Wiley & Sons, Hoboken.
Grace TDC (1962), Establishment of four strains of cells from insect tissues grown in vitro, Nature 195:788 789.
Ham RG (1965), Clonal growth of mammalian cells in a chemically defined synthetic medium, Proc. Natl Acad. Sci. U S A 53:288.
Hasegawa A, Yamashita H, Kondo S, Kiyota T, Hayashi H, Yoshizaki H, Murakami A, Shiratsuchi M, Mori T (1988), Proteose peptone enhances production of tissue type plasminogen activator from human diploid fibroblasts, Biochem. Biophys. Res. Commun. 150:1230 1236.
Heidemann R, Zhang C, Qi H, Rule JL, Rozales C, Park S, Chuppa S, Ray M, Michales J, Konstantinov K, Naveh D (2000), The use of peptones as medium additives for the production of a recombinant therapeutic protein in high density

perfusion cultures of mammalian cells, Cytotechnology 32:157 167.
Ikonomou L, Bastin G, Schneider YJ, Agathos SN (2001), Design of an efficient medium for insect cell growth and recombinant protein production, In Vitro Cell. Dev. Biol. Anim. 37:549 559.
Ikonomou L, Schneider YJ, Agathos SN (2003), Insect cell culture for industrial production of recombinant proteins, Appl. Microbiol. Biotechnol. 62:1 20.
Jenkins N (1991), Growth factors. In: Butler M (Ed.), Mammalian Cell Biotechnology, A Practical Approach, 1st Ed., IRL Press, Oxford, pp. 39 55.
Keay L (1976) Autoclavable low cost serum free cell culture media: the growth of established cell lines and production of viruses, Biotechnol. Bioeng. 18:363 382.
Maiorella B, Inlow D, Shauger A, Harano D (1998), Large scale insect cell culture for recombinant protein production, Bio/Technology 6:1406 1410.
Maranga L, Mendonça RZ, Bengala A, Peixoto CC, Moraes RHP, Pereira CA, Carrondo MJT (2003), Enhancement of Sf 9 cell growth and longevity through supplementation of culture medium with hemolymph, Biotechnol. Prog. 19: 58 63.
Mendonça RZ, Palomares LA., Ramírez OT (1999), An insight into insect cell metabolism through selective nutrient manipulation, J. Biotechnol. 72:61 75.
Mendonça RZ, de Oliveira E C, Pereira CA, Lebrun I (2007), Effect of bioactive peptides isolated from yeastolate, lactalbumin and NZCase in the insect cell growth, Bioprocess Biosyst. Eng. 30:157 164.
Mitsuhashi J (1989), Nutritional requirements of insect cells in vitro, In: Mitsuhashi J (Ed.), Invertebrate Cell System Applications, CRC Press, Boca Raton, pp. 3 20.
Mizrahi A (1977), Primatone® RL in mammalian cell culture media, Biotechnol. Bioeng. 19:1557 1561.
Montgomery DC, Runger GC (1999), Applied Statistics and Probability for Engineers, 2nd Ed., Wiley, New York.
Moore GE, Gerner RE, Franklin HA (1967), Culture of normal human leukocytes, J. Am. Med. Assoc. 199:519 524.
Moses HL, Coffey RJ Jr, Leof EB, Lyons RM, Keski Oja J (1987), Transforming growth factor beta regulation of cell proliferation, J. Cell Physiol. Suppl., Suppl. 5:1 7.
Öhman L, Ljunggren J, Haggstrom L (1995), Induction of a metabolic switch in insect cell by substrate limited fed batch cultures, Appl. Microbiol. Biotechnol. 43:1006 1013.
Parker RC, Castor LN, McCulloch EA (1957), Altered cell strains in continuous culture, Special Publications NY Acad. Sci. 5:303 313.
Rodrigues MI, Iemma AF (2005), Planejamento de Experimentos e Otimização de Processos: uma Estratégia Seqüencial de Planejamentos, 1st ed., Editora Casa do Pão, Campinas.
Schlaeger EJ, Foggetta M, Vonach JM, Christensen K (1993), SF 1 a low cost culture medium for the production of recombinant proteins in baculovirus infected insect cells, Biotechnol. Tech. 7:183 188.
Schlaeger EJ (1996), The protein hydrolysate, Primatone® RL, is a cost effective multiple growth promoter of mammalian cell culture in serum containing and serum free media and displays anti apoptosis properties, J. Immunol. Methods 194: 191 199.
Shuler ML, Kargi F (2002), Bioprocess Engineering Basic Concepts, 2nd Ed., Prentice Hall PTR, Upper Saddle River.
Slabicky R (1994), Pharmaceutical water systems: design and validation. In: Lydersen BK, D'Élia NA, Nelson KL (Eds), Bioprocess Engineering: Systems, Equipment and Facilities, John Wiley & Sons, New York, pp. 525 573.
Souza APB, Peixoto CC, Maranga L, Carvalhal AV, Moraes RHP, Mendonça RZM, Pereira CA, Carrondo MJT, Mendonça RZ (2005), Purification and characteriza

tion of an anti apoptotic protein isolated from Lonomia obliqua hemolymph, Biotechnol. Prog. 21:99 105.
Sporn MB, Roberts A, Wakefield LM, Crombrugghe (1987), Some recent advances in the chemistry and biology of transforming growth factor beta, J. Cell. Biol. 105:1039 45.
Sung YH, Lim SW, Chung JY, Lee GM (2004), Yeast hydrolysate as a low cost additive to serum free medium for the production of human thrombopoietin in suspension cultures of Chinese hamster ovary cells, Appl. Microbiol. Biotechnol. 63:527 536.
Taub N, Livingston D (1981), The development of serum free hormone supplemented media for primary kidney cultures and their use in examining renal functions, Ann. N Y Acad. Sci. 372:406 21.
Van der Valk J, Mellor D, Brands R, Fischer R, Gruber F, Gstraunthaler G, Hellebrekers L, Hyllner J, Jonker FH, Prieto P, Thalen M, Baumans V (2004), The humane collection of fetal bovine serum and possibilities for serum free cell and tissue culture, Toxicol. Vitro 18:1 12.
Wu J, Lee KD (1998), Growth promotion by yeastolate and related components on insect cells, Biotechnol. Tech. 12:67 70.
Wu J, Ruan Q, Peter Lam HY (1998), Evaluation of spent medium recycle and nutrient feeding strategies for recombinant protein production in the insect cell baculo virus process, J. Biotechnol. 66:109 116.

6 Post-translational modification of recombinant proteins

Michael Butler

6.1 Introduction

Many proteins require post-translational modifications to insure the full biological activity that is essential for their use as efficacious biopharmaceuticals. The most widely recognized form of post-translational modification is glycosylation that requires an extensive sequence of processing and trimming events in the endoplasmic reticulum (ER) and Golgi apparatus. Because eukaryotic cells have the metabolic capability for these types of modifications they have become the host cells used in biopharmaceutical processes. More specifically, mammalian cells (particularly the hamster BHK and CHO cells) have become the host cells of choice because the glycosylation patterns generated from these cells are most similar to equivalent human patterns. Most therapeutic proteins are glycoproteins that have an extensive heterogeneity of glycoforms arising from incomplete glycosylation processes.

Although glycosylation is the most extensive and widely studied form of post-translational modification, there are other modifications that are equally important for the activity of specific proteins. The blood clotting factor, factor X, is a good example to illustrate a range of post-translational modifications on a single complex protein. The recognized modifications necessary to ensure bioactivity of this factor include: removal of signal sequences, formation of disulfide bonds, gamma-carboxylation of glutamic acid residues, modification of aspartate to beta-hydroxyaspartate, N- and O-glycosylation, removal of an internal tripeptide, and removal of a propeptide (Camire *et al.*, 2000). In order to express this protein as a recombinant form in a cell culture bioprocess, it is necessary to insure that the host cell has the appropriate metabolic capabilities for efficient post-translational modification and also to insure that the culture conditions are suitable to drive the metabolism to high productivity of the active protein.

This chapter describes some of the most important and well-studied forms of post-translational modifications of proteins, which have been associated with the production of glycoproteins as biopharmaceuticals from mammalian cell culture systems. Most attention is given to glycosylation because of its general importance for the activity of all these

proteins. However, other modifications are also described and related to the production of specific proteins.

6.2 Glycan structures attached to proteins

Carbohydrate chains when attached to other molecules are known as glycans. In proteins the glycans can be attached to asparagine residues forming N-glycans or to serine or threonine residues forming O-glycans.

6.2.1 N-glycans

This is the most widely studied structural form of glycosylation and has the greatest effect on overall protein structure and function (Kornfeld and Kornfeld, 1985; Spellman, 1990). The glycan consists of a core pentasaccharide ($Man_3GlcNAc_2$) attached via an N-glycosidic bond to an Asn residue of a consensus amino acid sequence (sequon) Asn-X-Ser/Thr, where X can be any amino acid except proline (*Figure 6.1*).

The full sequence of reactions involved in the synthesis of the N-glycans in mammalian cells is shown in *Figure 6.2*. The precursor for glycosylation is the oligosaccharide ($Glc_3Man_9GlcNAc_2$), which is formed by the stepwise addition of monosaccharides from nucleotide sugars (UDP-GlcNAc and GDP-Man) or lipid intermediates (Dol-P-Man and Dol-P-Glc) to a dolichol-linked pyrophosphate acceptor to form the complex: $Glc_3Man_9GlcNAc_2$-P-P-dolichol. The 14-mer oligosaccharide is transferred to the consensus sequence (Asn-X-Ser/Thr) on the growing peptide via the complex reaction of the oligosaccharyltransferase (OST) enzyme, which is thought to be approximately 65 amino acid residues from the ribosome P-site (Yan and Lennarz, 2005). Further processing by trimming of the attached glycan occurs by glucosidases and mannosidases that are

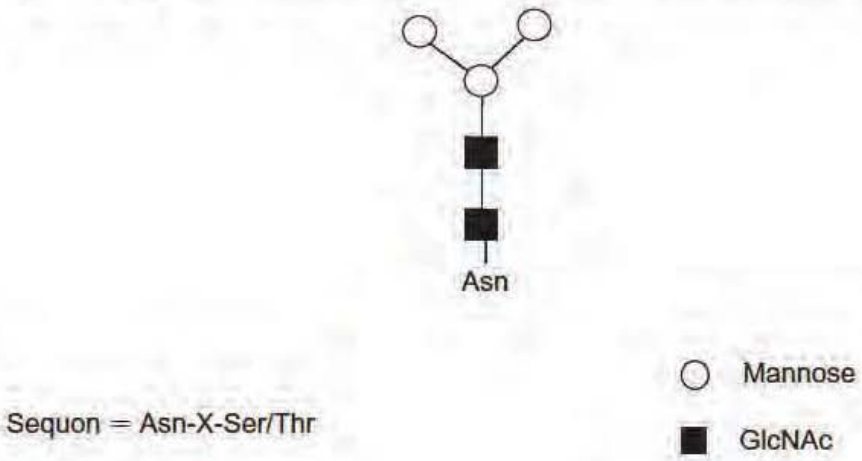


Figure 6.1

Core N glycan structure. This consists of a core pentasaccharide composed of a trimannosyl group attached to two Glc NAc residues. The structure is attached to an asparagine (Asn) residue in a sequon which is an amino acid motif of three amino acids. X is any amino acid (except Pro) that is positioned between Asn and either serine or threonine.

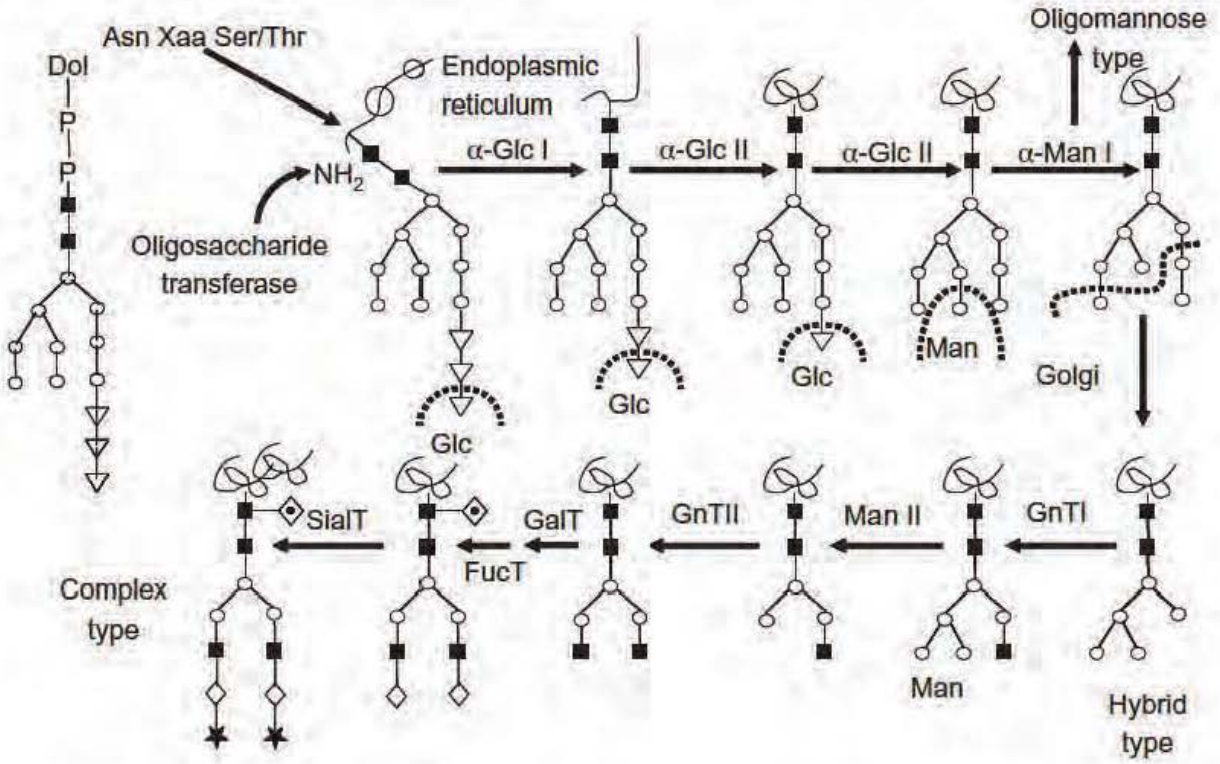


Figure 6.2

N linked glycosylation pathway. The addition of a 14mer oligosaccharide to an N glycan sequon occurs co translationally in the endoplasmic reticulum. This is followed by a series of trimming reactions to remove glucose and mannose residues. In animal cells the glycan structures with exposed mannose groups and attached to newly synthesised proteins are transferred to the Golgi for further processing. A series of transferase enzymes ensures the addition of N acetylglucosamine, fucose, galactose, and sialic acids. The transferase reactions form a metabolic network resulting in a variety of end products, which account for the variable glycoforms found in proteins.

located within the membrane of the ER. Trimming is initiated by α-1,2 glucosidase I (Gluc I) which removes the terminal glucose. The next two glucose residues are then removed sequentially by a single α-1,3 glucosidase II (Gluc II). At least one mannose residue may then be removed by mannosidase I (Man I) before the newly synthesized glycoprotein is transported to the Golgi cisternae by means of vesicles. This sequential process of trimming in the ER will only occur efficiently if the protein assumes an appropriate folding pattern and so the process has been thought of as a quality control mechanism required for the formation of the protein tertiary structure (Ellgaard and Helenius, 2003).

The core structure may be processed further to a complex-type glycan with the sequential addition of monosaccharides such as GlcNAc, galactose, fucose, and sialic acid through a series of transferases that are present in the Golgi. In mammalian cells high-mannose glycan structures are produced as intermediates in the formation of complex glycans (*Figure 6.3*). These may have two to six additional mannose structures attached to the core as a result of processing in the ER prior to translocation to the

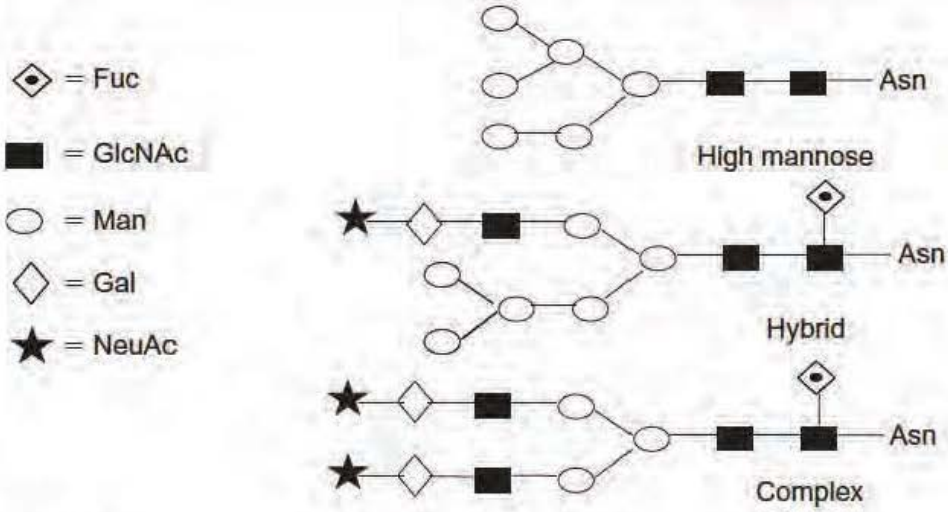


Figure 6.3

N glycan structures of glycoproteins. There are three main types of glycan structure, which are built on the core structure. The high mannose structures are the main type of structures found in fungi. In animal cells these are generally intermediates that are transferred to the Golgi for further processing. The complex glycans consist of a variety of structures added in sequence to the trimannosyl core. The hybrid structures consist of at least one antenna from the trimannosyl core with a complex structure and one with a high mannose structure.

Golgi. However, in lower eukaryotes such as fungi and yeast the high-mannose structures are released from the cell as end products of glycosylation (Meynial-Salles and Combes, 1996).

There is a great deal of variability of N-glycan structures attached to any protein. This arises through macroheterogeneity, which is defined as variable occupancy of potential sites of attachment and microheterogeneity, which is defined as variable structural forms at each site.

6.2.1.1 Macroheterogeneity

The occupation of a sequon occurs cotranslationally in the ER and involves the transfer of a consensus oligosaccharide structure ($Glc_3Man_9GlcNAc_2$) from a dolichol-linked pyrophosphate donor to the Asn side chain of an available sequon. Only around 65% of all appropriate glycoprotein sequons are occupied and the oligosaccharyl transfer reaction appears to depend upon various cellular factors including the protein translation rate, the availability of the oligosaccharyl donor, and the activity of the enzyme. Petrescu *et al.*, (2004) surveyed the occupation of sequons on several thousand proteins and found a significantly large number of occupied glycans that have low accessibility in the folded protein. Although this may seem surprising, it must be realized that protein glycosylation precedes folding and so the inaccessibility of a sequon in the fully folded protein does not mean that it is necessarily inaccessible to the OST enzyme. The glycans may play a part in maintaining the folded structure by reducing the conformational freedom of the local peptide backbone (Petrescu *et al.*, 2004). However, disulfide bridge

formation is also co-translational in the ER and may interfere with the accessibility of some sequons, leaving them unoccupied. For some proteins such as tissue-type plasminogen activator (tPA) this may lead to variable occupancy of a specific site and macroheterogeneity of glycoforms (Allen *et al*., 1995).

A further consideration is the position of the sequon in relation to the primary structure of the protein. Statistical analysis of a large number of glycoproteins has indicated that the frequency of non-glycosylated sequons increases toward the C-terminus (Gavel and von Heijne, 1990). The critical distance appears to be 60 amino acid residues from the C-terminus when reduced glycan occupation occurs. This distance corresponds to the distance between the ribosome P-site and the active site of the OST and it has been hypothesized that the protein chain is not available for N-glycan attachment once it is released from the ribosome. However, this phenomenon of poor glycosylation efficiency toward the C-terminus does not appear to be universal for all proteins (Walmsley and Hooper, 2003).

6.2.1.2 Microheterogeneity

The transferase reactions in the Golgi do not always go to completion and so give rise to heterogeneity of the final glycan structure (microheterogeneity). This heterogeneity is evident as variable antennarity, with the number of branches from the central mannose of the core structure ranging typically between two (biantennary) and four (tetra-antennary). Terminal sialylation of the antenna, fucosylation of the innermost core GlcNAc (proximal) or the outer arm GlcNAc (peripheral), and addition of a ''bisecting'' GlcNAc to the central core Man residue are also examples of glycan processing that is variable and may be incomplete in the Golgi.

A diverse range of glycan forms may be produced in some recombinant proteins. For example, the HIV envelope glycoprotein (gp120) produced from CHO cells contains 24 occupied sequons, of which 13 are complex glycans and 11 are high mannose or hybrid structural forms (Leonard *et al.*, 1990).

6.2.2 O-linked glycans

Although most attention has been focused on N-linked glycosylation of proteins, O-linked glycans are smaller structurally but equally ubiquitous among eukaryotic glycoproteins. The most common form of O-glycan attached to glycoproteins from mammalian cells is the mucin-type, which involves the addition of N-acetylgalactosamine to a serine or threonine residue in a protein. The O-glycan is added post-translationally to the fully folded protein. No consensus sequence has been identified, although glycosylation often occurs in a region of the protein that contains a high proportion of serine, threonine, and proline (Van den Steen *et al.*, 1998). These residues probably enable the region of the protein to assume a conformation that is accessible to the GalNAc transferase enzyme responsible for the addition of the GalNAc. The first step for the assembly of the mucin type O-glycans is the addition of N-acetylgalactosamine (GalNAc) residue to a Ser/Thr by a GalNAc transferase (GalNAcT) from UDP-

GalNAc (Van den Steen *et al.*, 1998). Further elongation leads to a large number of structures, synthesized by various glycosyltransferases, producing eight different core structures (*Figure 6.4*). These core structures can be further modified by sialylation, fucosylation, sulfation, methylation, or acetylation. The most common is the core 1 structure (Galβ1-3GalNAc) that may be monosialylated or disialylated. These are the most prominent O-glycan structures found in glycoproteins produced from CHO cells (Backstrom *et al.*, 2003).

6.2.3 Patterns of glycosylation in non-mammalian cells

The prokaryotes (mainly *E. coli*) were the first cells to be used for gene expression of recombinant proteins. These cells can be manipulated genetically and show high productivity in large-scale systems. However, the cells lack any metabolic pathways for glycosylation and so the proteins produced are not glycosylated. Lower eukaryotes (yeast, insect, and plant cells) are capable of glycosylation of proteins. However, the glycans produced in these cells differ significantly from those present in mammalian glycoproteins (Jenkins *et al.*, 1996). Yeast, insect, plant, and mammalian cells share the feature of N-linked oligosaccharide processing in the ER, including attachment of $Glc_3Man_9GlcNAc_2$-P-P-Dol and subsequent truncation to $Man_8GlcNAc_2$ structure. However, oligosaccharide processing by these different cell types diverges in the Golgi apparatus (Goochee

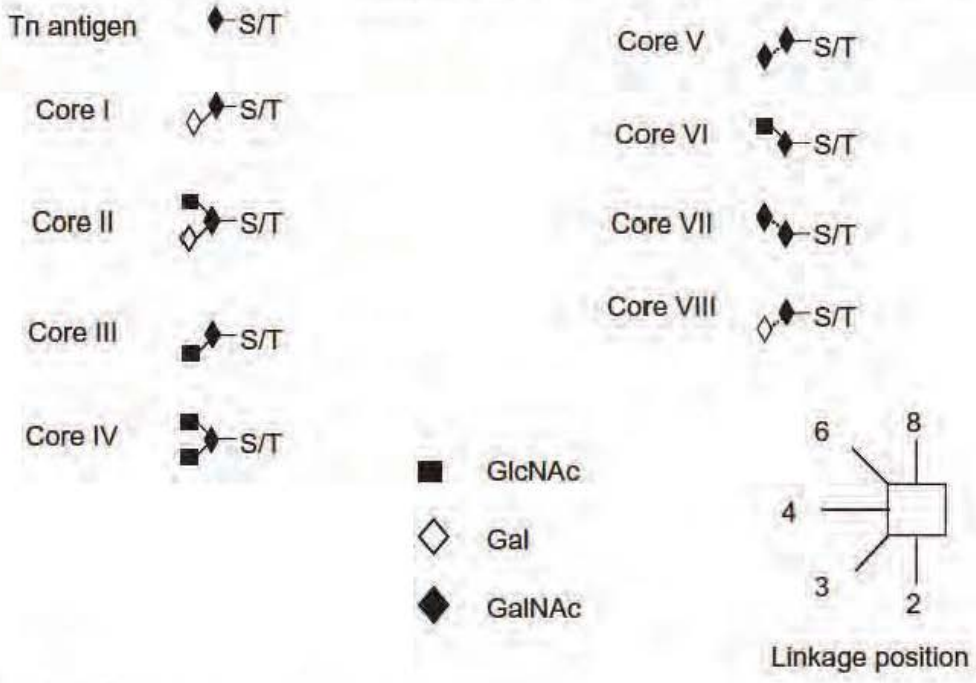


Figure 6.4

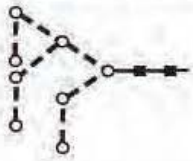
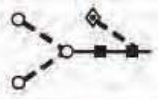
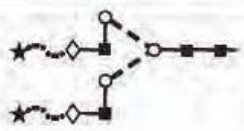
Core structures of mucin type O linked glycans. These represent a series of characteristic structures attached to a serine (S) or threonine (T) amino acid residue of a protein. There are eight core structures, each of which may have further monosaccharide additions. Tn is a precursor of the T (Thomsen Freidenreich) antigen which is commonly used as a marker for tumor cells.

et al., 1991). Although there is extensive heterogeneity of structures arising from any cell type, examples of predominant N-glycans that might occur from different systems are shown in *Table 6.1*.

Insect cells. Lepidopteran insect cell lines (such as *Spodoptera frugiperda, Sf-9*) have been used extensively for expression of recombinant proteins using the baculovirus as an expression vector (Jarvis *et al.*, 1998). Alternative methods of protein expression are also available using efficient insect-associated promoter systems (Farrell *et al.*, 1998). The advantage of the use of these cells is the high expression level and growth rate of the cells in culture. However, the glycosylation of proteins expressed by insect cells is limited. These cells can add $Glc_3Man_9GlcNAc_2$ precursors to appropriate N-glycan sites in a nascent polypeptide and convert them to $Man_9GlcNAc_2$. They also have the enzymes necessary to trim this oligosaccharide all the way down to $Man_3GlcNAc_2$. The formation of this product requires the action of the enzymes ManI, GnTI, and ManII. However, there is little structural processing beyond the $Man_3GlcNAc_2$ core oligosaccharide apart from the possibility of fucosylation (Jarvis and Finn, 1996; Donaldson *et al.*, 1999).

Glycosyltransferase enzymes are either absent or at a low level of activity (Jarvis *et al.*, 1998). Therefore, generally the insect expression system is incapable of synthesizing sialylated lactosamine complex-type N-glycan or sialylated O-glycans. However, some insect cells have been found to produce recombinant glycoproteins with elongated trimannosyl core structures containing terminal GlcNAc or Gal, and one recombinant glycoprotein acquired complex biantennary N-linked glycan containing sialic acid (Davidson *et al.*, 1990; Kulakosky *et al.*, 1998).

Table 6.1 Typical predominant N-glycan structures from different cell types.

Cell type	N glycan	Structure
Bacteria, *E.coli*	None	-
Yeast	High mannose	
Insect	Fucosylated core structure	
Plant	Xylosylated and fucosylated core structure	
Mammalian	Complex biantennary	

Jarvis *et al*. (1998) proposed a model to explain the N-linked oligosaccharide structures found in insect cell-produced glycoproteins**.** In this model the enzyme GlcNAcTII competes with N-acetylglucosaminidase (GlcNAdase) at a branch point. Depending upon the relative activities of these competing processing enzymes, trimannosyl core or complex N-linked glycans may be produced. It would appear that insect cells have a high level of N-acetylglucosaminidase activity, which can remove GlcNAc from a terminal position. The production of complex sialylated N-linked structures could only occur when the recombinant protein is a very poor substrate for GlcNAdase or excellent substrate for the low level activities of the glycosyltransferases. The enzymic activity may vary considerably and this explains the differences in the N-glycan pattern of the same glycoprotein secreted by different insect cell lines (Kulakosky *et al*., 1998).

The potential of insect cells for O-glycosylation was reported in a study in which three lepidopteran cell lines were shown to produce predominantly short O-glycan structures (Lopez *et al.*, 1999). All three cell lines expressed GalNAcα1-O-Ser/Thr (Tn antigen), whereas the ability to synthesize Galβ1-3GalNAcα1-O-Ser/Thr (T antigen) and Galα1-4Galβ1-3GalNAcα1-O-Ser/Thr (PT antigen) was more limited. This indicated low activity of β1-3-galactosyltransferase and α1-4-galactosyltransferase. There was no indication of sialylation of these structures, suggesting the absence of sialyltransferase activity. The culture medium used to grow these cell lines had a major effect on the O-glycans expressed. The use of a semi-defined rich medium enhanced glycosyltransferase activity significantly compared with a minimal nutrient medium. The limitations in the ability of insect cells for glycosylation have restricted their use for production of human therapeutics. Genetic engineering of these systems is under study in an attempt to improve the glycosylation machinery to produce more ‘‘humanized’’ glycoproteins (Jarvis *et al*., 1998). In particular the transformation of insect cells with the gene for mammalian β1,4-N-acetylgalactosyltransferase with a baculovirus expression vector leads to expression of the enzyme and results in more extensively processed N-glycans (Jarvis and Finn, 1996).

Fungi. The early steps in the addition of carbohydrate to proteins appear to have been remarkably conserved during evolution. The synthesis of the $Glc_3Man_9GlcNAc_2$-P-P-Dol precursor, the transfer to the polypeptide, and early processing in the ER are common events shared by eukaryotic cells. However, although relatively few N-linked sites have been characterized, it was noted that there is a trend in favor of the use of Asn-X-Thr sites over Asn-X-Ser in yeast glycoproteins. In contrast to mammalian cells, where several Man residues may be removed during processing, in *Saccharomyces cerevisiae*, a single specific Man residue is cleaved to form $Man_8GlcNAc_2$. Most yeast and filamentous fungi synthesize carbohydrate chains of the high mannose type. Complex glycan structures are not observed among fungal glycoproteins. Addition of Man residues to core oligosaccharides occurs very rapidly in the Golgi, forming the characteristic high mannose structures (mannan), which can consist of more than 50 mannose residues and resulting in high molecular weight glycoproteins (Hersecovics and Orlean, 1993).

Proteins synthesized in yeast may also contain O-glycans consisting of linear poly-mannose structures attached to Ser or Thr. Similar to mammalian cells, O-glycosylation in yeasts has no obvious consensus sequence. However, unlike mammalian cells O-glycosylation in yeast is initiated with covalent attachment of mannose via a dolichol phosphate mannose precursor. Maras *et al*., (1997) showed that if the high mannose structures are trimmed *in vitro* by mannosidase, they can become acceptors for the recombinant processing enzymes, N-acetylglucosaminyltransferase I, β1,4-galactosyltransferase, and α2,6-sialyltransferase. Mutant strains of yeast also may synthesize truncated mannose structures.

Plants. Plant cells also conserve the early stages of N-glycosylation. However, the processing of the oligosaccharide trimming and further modification of glycans in the Golgi differ from mammalian cells. Plant-derived oligosaccharides do not possess sialic acid and frequently contain xylose (Xyl), not normally present in mammalian N-linked oligosaccharides. Typically processed N-glycans in plants have a $Man_3GlcNAc_2$ structure with β1,2 xylose and/or α1,3 fucose residues attached to the reducing terminal GlcNAc (Palacpac *et al*., 1999). Xylose is not present in mammalian glycan structures and fucose is attached to proximal (core) GlcNAc by an alternative linkage (α1,6) in mammalian cells. The presence of these two residues (Xyl and α1,3 fucose) in plant recombinant glycoproteins and their absence in mammalian proteins makes them highly immunogenic if present in therapeutic glycoproteins (Parekh *et al*., 1989; Storring, 1992; Palacpac *et al*., 1999).

6.2.4 Glycosylation in animal cells: the effect of the host cell line

The pattern of protein glycosylation is dependent on the expression of various glycosyltransferase enzymes that are present in the Golgi of the cell. Differences in the relative activity of these enzymes among species can account for significant variations in structure. In one systematic study of glycan structures of IgG produced from cells of 13 different animal species significant variation was found in the proportion of terminal galactose, core fucose, and bisecting GlcNAc (Raju *et al.*, 2000).

The fact that glycoproteins normally exist as mixtures of glycoforms suggests that the protein structure is not the primary determining factor in glycosylation. The glycoforms that emerge from the Golgi are end products of a series of incomplete enzymic reactions. Thus, the choice of the host cell line is a particularly important factor in the glycoform profile of a recombinant protein (Rudd and Dwek, 1997). Sialylation patterns of the secreted protein are particularly affected by the host cell.

6.2.5 Culture parameters that may affect glycosylation

It is important to control culture parameters to insure consistency of glycosylation of a recombinant protein in a culture bioprocess. However, this may not be so easy given that the extent of glycosylation may decrease over time in a batch culture (Curling *et al.*, 1990). This is likely to be due to the continuous depletion of nutrients (particularly glucose or glutamine) and accumulation of metabolic byproducts, which have been shown

to limit the glycosylation process (Hayter *et al.*, 1992; Jenkins and Curling, 1994; Nyberg *et al.*, 1999). Culture conditions such as nutrient content, pH, temperature, oxygen, or ammonia, may have a significant impact on the distribution of glycan structures found on the resulting recombinant protein (microheterogeneity). This of course is of major concern in trying to produce consistent biopharmaceuticals. It can lead to enhanced glycoform heterogeneity and significant batch to batch variation in the production process. To maintain product quality it is important to understand the parameters that cause the variation in glycosylation.

Glucose starvation may result in an intracellular depleted state with a shortage of glucose-derived precursors of glycans that could result in reduced site occupancy. This has been shown at low glucose concentration (< 0.5 mM) for the synthesis of antibody (Stark and Heath, 1979) and recombinant gamma-interferon (Hayter *et al.*, 1992). Fed-batch strategies may be designed to insure that the concentrations of these key nutrients do not decrease below a critical level that could compromise protein glycosylation (Xie and Wang, 1997). The lower levels of nutrients for the production of recombinant protein from CHO cells were found to be 0.1 mM glutamine and 0.7 mM glucose (Chee *et al.*, 2005). Nutrient levels below these critical concentrations led to decreased sialylation and an increase in hybrid and high mannose-type glycans. A plausible mechanism for reduced site occupancy of a recombinant protein produced by glucose-depleted or glutamine-depleted CHO cell cultures was offered by Nyberg *et al.* (1999). They showed that in both cases the low level of glycosylation was related to a decreased intracellular concentration of UDP-GlcNAc.

This intracellular concentration of UDP-GlcNAc has also been shown to be important in another respect. An elevated UDP-GlcNAc appears to cause a decrease in sialylation, which may be explained metabolically by the inhibition of CMP-sialic acid transport (Pels Rijcken *et al.*, 1995). This may be the mechanism for the observed decrease in sialylation that has been shown to accompany high levels of ammonia in culture (Yang and Butler, 2000).

The dissolved oxygen (DO) level of cultures has also been shown to be a critical parameter to affect glycosylation. Terminal galactosylation of an immunoglobulin (IgG) was changed significantly with a gradual decrease in the digalactosylated glycans (G2) from 30% at the higher oxygen level to around 12% under low oxygen conditions (Kunkel *et al.*, 1998).

6.3 Other forms of post-translational modification

6.3.1 Deamidation

Non-enzymatic deamidation of glutamine or asparagine residues of proteins can occur spontaneously, leading to potential structural changes and loss of biological activity. Deamidation of Asn occurs more rapidly and leads to α-linked (Asp) or β-linked aspartyl (Isoasp) via a succinamide derivative. This introduces an extra negative charge in the protein that can result in alterations of properties. For example, deamidation of an Asn residue of human growth hormone-releasing factor can lead to a 25–500-

fold loss in potency (Friedman *et al.*, 1991). Low levels of deamidation can lead to protein aggregation and are related to amyloid formation (Nilsson *et al.*, 2002). The presence of Isoasp may also enhance the immunogenicity of a protein. Deamidation may occur during bioprocessing or storage of biopharmaceuticals and is dependent upon a number of factors including pH, temperature, solvent dielectric constant, and ionic strength, as well as the structure of the protein. Deamidation half-times for Asn in synthetic peptides at neutral pH and 37°C are in the range of 1–500 days. Huang *et al.* (2005) described ''hot spots'' for spontaneous deamidation in a humanized monoclonal antibody (mAb) that can lead to loss of binding capacity.

6.3.2 Deamination

Proteins subjected to metal-catalyzed oxidation can form carbonyl groups that are essentially deamination products (glutamic and aminoadipic semialdehydes) that result from proline, arginine and lysine residues. This may lead to loss of function or structural alterations of the proteins that are associated with the loss of a positive charge. Glutamic semialdehyde (5 mmole/mol) accounted for the majority of carbonyl groups in proteins from HeLa cells in culture. Treatment of the cells with an H_2O_2-generating system led to a 2.5-fold increase of these carbonyl groups (Requena *et al.*, 2001).

Oxidative deamination of lysine has also been observed by incubating a model protein in the presence of high concentrations of glucose (50 mM) or lower concentrations of the α-oxoaldehydes; methyl glyoxal, or 3-deoxyglucosone (1 mM; Akagawa *et al.*, 2002). These α-oxoaldehydes may be formed by the nonenzymatic degradation of glucose or from the early stage of glycation (Thornalley *et al.*, 1999). The presence of copper (Cu^{2+}) increases the rate of this process.

6.3.3 Glycation

This is a non-enzymatic process of adding glycans that may contribute to the post-translational modification of proteins. It is a complex process that results in lysine- and arginine-derived glycation adducts in intracellular or extracellular proteins. The initial Maillard reaction involves the formation of a Schiff's base between glucose and the ε-amino group of lysine (*Figure 6.5*). This undergoes a rearrangement to form a more stable fructosamine–protein adduct. However, a number of subsequent reactions are possible that lead to advanced glycation end products (AGEs) that have been associated with pathological complications of diabetes. The process has been well studied in connection with pathological hyperglycemia. However, there are also some specific considerations for bioprocesses.

Biopharmaceuticals may be subjected to heat treatment as a means of viral inactivation. Protein damage is prevented by the addition of high concentrations of a thermostabilizing excipient such as sugars or polyhydric alcohols. The non-reducing polyhydric additives increase thermostability of the protein by the formation of a hydrogen-bonded solvent shell. The typical treatment for factor VIII consists of heat treatment at 60°C for

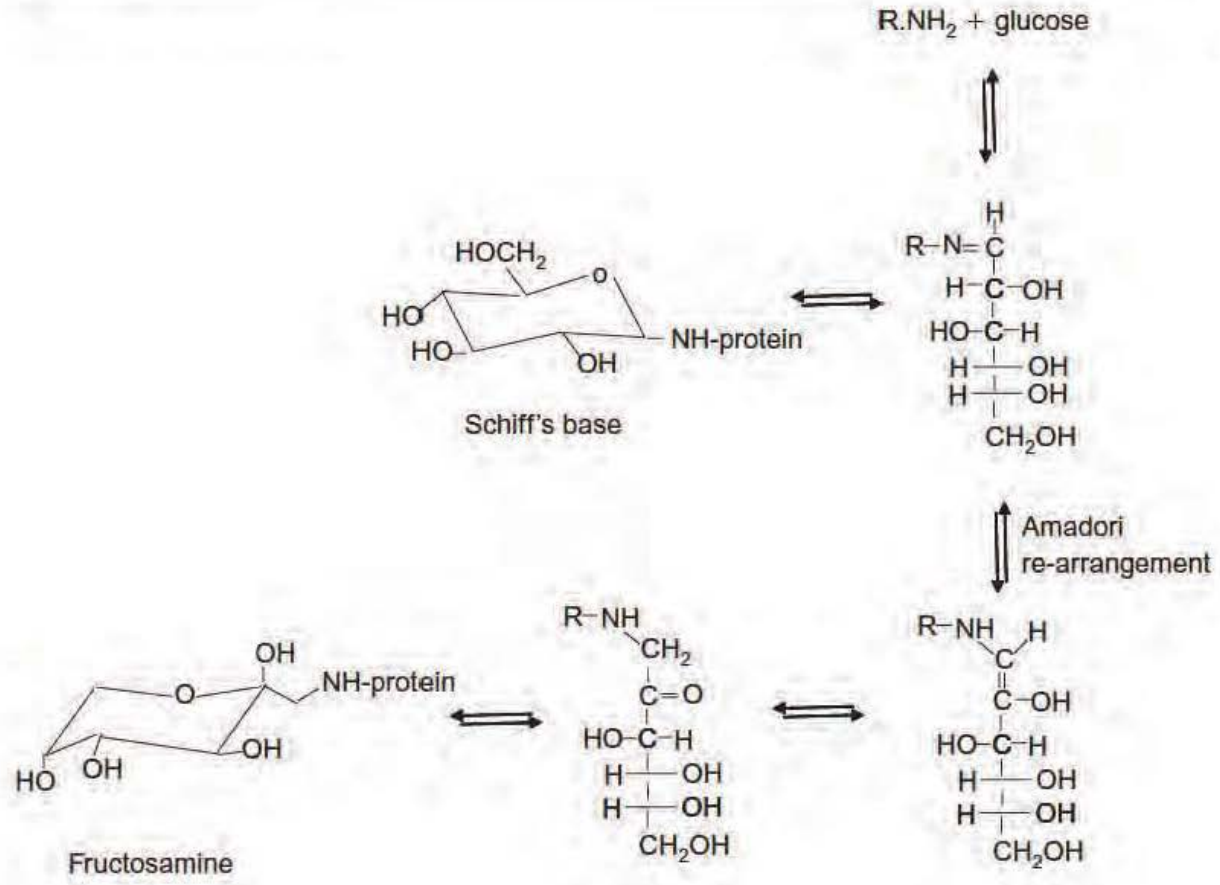


Figure 6.5

Glycation reactions. Glucose or other sugars may react non enzymatically with proteins to form glycation products. Early glycation products such as fructosamine protein adducts may later be transformed to the more complex advanced glycation end products (AGE products).

10 h in the presence of high concentrations of sucrose and glycine. Such a treatment will destroy most viruses but may cause glycation products that could render the protein immunogenic (Smales *et al.*, 2002). The observed modifications take place by reaction adducts of glucose or fructose that are derived from the hydrolysis of sucrose. The heat treatment can result in deamidation, protein glycation, or AGE adduct formation.

6.3.4 Gamma-carboxylation

Gamma-carboxyglutamic acid (Gla) is an amino acid with a dicarboxylic acid side chain that has metal-binding properties and is important for the biological activities of a range of blood coagulation proteins that include prothrombin, factor X, factor IX, and factor VII, as well as the regulatory proteins, protein C and protein S. The extra negative charge provided by the carboxylation aids in the calcium-induced interaction of these proteins with membrane surfaces. Gamma-carboxyglutamic acid is synthesized by the post-translational modification of glutamic acid residues (Glu) in a reaction catalysed by a carboxylase enzyme with requirements for vitamin

K, O_2, and CO_2 (*Figure 6.6*). The carboxylation of glutamic acid residues of these proteins appears to be directed by a specific recognition sequence, which is often found in the propeptide to insure binding to the vitamin K-dependent carboxylase. Furie *et al.*, (1999) identified five requirements for gamma carboxylation:

(i) there is a recognition site that interacts with the carboxylase enzyme;
(ii) the protein is trafficked through the rough ER during synthesis;
(iii) the cell has a carboxylase enzyme associated with the rough ER;
(iv) there are Glu residues within 40 residues of the enzyme recognition sequence;
(v) intracellular vitamin K is present.

The production of recombinant protein C has been studied from BHK cells and it was shown that the protein secretion rate increased with cDNA copy number. However, a higher secretion rate decreased the efficiency of gamma-carboxylation (Guarna *et al.*, 1995). Similarly, culture conditions were shown to affect carboxylation of protein C, with a good supply of oxygen favoring high efficiency of gamma-carboxylation (Sugiura and Maruyama, 1992).

Factor X undergoes extensive post-translational modification including gamma-carboxylation of 11 glutamic acid residues. However, the efficiency of this process is variable and inevitably leads to a heterogeneity of isoforms. HEK-293 cells have been shown to be more efficient for gamma-carboxylation than CHO cells. This may depend on the availability and activity of the carboxylase enzyme that binds to the propeptide. Camire *et al.*, (2000) improved the efficiency of carboxylation of factor X by using a chimeric cDNA that contained a propeptide with reduced affinity to the enzyme. Surprisingly, this increased the extent of carboxylation from a normal value of 32% to 85% by enhancing substrate turnover. Nevertheless, in all situations there were two pools of protein produced: a fully carboxylated and uncarboxylated recombinant factor X.

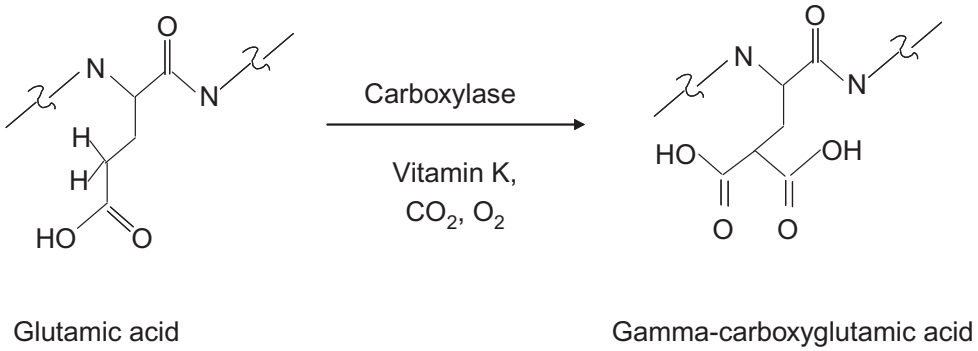


Figure 6.6

Gamma carboxylation of glutamic acid residue in a protein. This is an enzymic reaction catalyzed by a carboxylase to modify a glutamic acid residue of a protein to form gamma carboxyglutamic acid. The carboxylase reaction requires vitamin K, O_2, and CO_2.

6.3.5 C-terminal modifications

A cause of heterogeneity in some proteins arises from the partial cleavage of terminal lysine or arginine by an intracellular carboxypeptidase. For mAbs the C-terminal lysine of heavy chains may be removed, giving rise to a mixture of Lys0 (absence) or Lys1 (presence) heavy chain variants. Analysis by mass spectrometry showed that the Lys0 variant can amount to 40–45% of the total (Lazar *et al.*, 2004). Removal of C-terminal arginine has been shown for human recombinant EPO and also for the two-chain form of tPA following zymogen activation (Harris, 1995). It is considered that the removal of either C-terminal lysine or arginine is unlikely to cause loss of biological activity because they are found as natural variants of these proteins under physiological conditions *in vivo*.

6.3.6 Hydroxylation

The most well-known example of protein hydroxylation arises from the activity of the enzyme, prolyl-4-hydroxylase. This enzyme can convert proline to hydroxyproline when the substrate is contained in an amino acid motif (X-Pro-Gly). This is known to occur in collagens and requires four co-substrates: ferrous ion, 2-oxoglutarate, oxygen, and ascorbate (Kivirikko *et al.*, 1989). An example of this type of conversion has also been found in the N-terminus of the prion protein in which there is a domain containing poly (L-proline) (Gill *et al.*, 2000). An asparagine and proline hydroxylase may serve to regulate the activity of a hypoxia-inducible factor (HIF) *in vivo* (Lando *et al.*, 2002). Under normoxic conditions these enzymes are active and are capable of hydroxylating specific asparagine and proline residues in HIF, which is then targeted for destruction via the ubiquitin proteasome pathway. However, hypoxia inhibits the activity of the enzymes, allowing the HIF to function as a transcriptional regulator of genes, whose products play a role in adapting for oxygen deficiency *in vivo*.

6.4 Conclusions

Many biopharmaceuticals are produced as secreted glycoproteins from mammalian cell culture. The post-translational modification of these proteins is essential to insure structural stability and biological and clinical activity. The best characterized of these modifications is N-glycosylation. This gives rise to a significant heterogeneity of structural forms and it is important to monitor these to insure consistency of production. However, the ability to control the glycosylation is limited by our understanding of the parameters that affect the heterogeneity of added glycan structures. It is clear that the glycosylation process is affected by a number of factors including the three-dimensional structure of the protein, the enzyme repertoire of the host cell, the transit time in the Golgi, and the availability of intracellular sugar-nucleotide donors. From a process development perspective there are many culture parameters that can be controlled to enable a consistent glycosylation profile to emerge from each batch culture. A further, but more difficult goal is to control the culture

conditions to enable the enrichment of specific glycoforms identified with desirable biological activities. There are also a number of other possible post-translational modifications of proteins that can be characterized as the addition or removal of small organic residues. These may be important for the structural integrity of the protein and should be monitored in culture bioprocesses designed for biopharmaceutical production.

Acknowledgment

The Natural Science and Engineering Research Council (NSERC) of Canada is gratefully acknowledged for financial support for the study of post-translational modification of proteins through a series of Discovery and Collaborative Research and Development (CRD) grants.

References

Akagawa M, Sasaki T, Suyama K (2002), Oxidative deamination of lysine residue in plasma protein of diabetic rats. Novel mechanism via the Maillard reaction, Eur. J. Biochem. 269:5451 5458.

Allen S, Naim HY, Bulleid NJ (1995), Intracellular folding of tissue type plasminogen activator. Effects of disulfide bond formation on N linked glycosylation and secretion, J. Biol. Chem. 270:4797 4804.

Backstrom M, Link T, Olson FJ, Karlsson H, Graham R, Picco G, Burchell J, Taylor Papadimitriou J, Noll T, Hansson GC (2003), Recombinant MUC1 mucin with a breast cancer like O glycosylation produced in large amounts in Chinese hamster ovary cells, Biochem. J. 376:677 686.

Camire RM, Larson PJ, Stafford DW, High KA (2000), Enhanced gamma carboxyla tion of recombinant factor X using a chimeric construct containing the prothrom bin propeptide, Biochemistry 39:14322 14329.

Chee F, Wong D, Tin Kam Wong K, Tang Goh L, Kiat Heng C, Gek Sim Yap M (2005), Impact of dynamic online fed batch strategies on metabolism, productivity and N glycosylation quality in CHO cell cultures, Biotechnol. Bioeng. 89: 164 177.

Curling EM, Hayter PM, Baines AJ, Bull AT, Gull K, Strange PG, Jenkins N (1990), Recombinant human interferon gamma. Differences in glycosylation and proteo lytic processing lead to heterogeneity in batch culture, Biochem. J. 272:333 337.

Davidson DJ, Fraser MJ, Castellino FJ (1990) Oligosaccharide processing in the expression of human plasminogen cDNA by lepidopteran insect (Spodoptera frugiperda) cells. Biochemistry. 29(23):5584 90.

Donaldson M, Wood HA, Kulakosky PC (1999), Glycosylation of a recombinant protein in the Tn5B1 4 insect cell line: influence of ammonia, time of harvest, temperature, and dissolved oxygen, Biotechnol. Bioeng. 63:255 262.

Ellgaard L, Helenius A (2003), Quality control in the endoplasmic reticulum, Nat. Rev. Mol. Cell Biol. 4:181 191.

Farrell PJ, Lu M, Prevost J, Brown C, Behie L, Iatrou K (1998), High level expression of secreted glycoproteins in transformed lepidopteran insect cells using a novel expression vector, Biotechnol. Bioeng. 60:656 663.

Friedman AR, Ichhpurani AK, Brown DM, Hillman RM, Krabill LF, Martin RA, Zurcher Neely HA, Guido DM (1991), Degradation of growth hormone releasing factor analogs in neutral aqueous solution is related to deamidation of asparagine residues. Replacement of asparagine residues by serine stabilizes, Int. J. Pept. Protein Res. 37:14 20.

Furie B, Bouchard BA, Furie BC (1999), Vitamin K dependent biosynthesis of gamma carboxyglutamic acid, Blood 93:1798 1808.
Gavel Y, von Heijne G (1990), Sequence differences between glycosylated and non glycosylated Asn X Thr/Ser acceptor sites: implications for protein engineering, Protein Eng. 3:433 442.
Gill AC, Ritchie MA, Hunt LG, Steane SE, Davies KG, Bocking SP, Rhie AG, Bennett AD, Hope J (2000), Post translational hydroxylation at the N terminus of the prion protein reveals presence of PPII structure in vivo, EMBO J. 19:5324 5331.
Goochee CF, Gramer J, Andersen DC, Bahr JB, Rasmussen JR (1991), The oligosac charides of glycoproteins: bioprocess factors affecting oligosaccharide structure and their effect on glycoprotein properties, Bio/Technology 9:1347 1355.
Guarna MM, Fann CH, Busby SJ, Walker KM, Kilburn DG, Piret JM (1995), Effect of cDNA copy number on secretion rate of activated protein C, Biotechnol. Bioeng. 46:22 27.
Harris RJ (1995), Processing of C terminal lysine and arginine residues of proteins isolated from mammalian cell culture, J. Chromatogr. A 705:129 134.
Hayter PM, Curling EMA, Baines AJ., Jenkins N, Salmon I, Strange PG, Tong JM, Bull AT (1992), Glucose limited chemostat culture of chinese hamster ovary cells producing recombinant human interferon . Biotechnol. Bioeng. 39:327 335.
Hersecovics A, Orlean P (1993), Glycoprotein biosynthesis in yeast. FASEB J. 7: 540 550.
Huang L, Lu J, Wroblewski VJ, Beals JM, Riggin RM (2005), In vivo deamidation characterization of monoclonal antibody by LC/MS/MS, Anal. Chem. 77:1432 1439.
Jarvis DL, Finn E (1996), Modifying the insect cell N glycosylation pathway with immediate early baculovirus expression vectors, Nat. Biotechnol. 14:1288 1292.
Jarvis DL, Kawar ZS, Hollister JR (1998), Engineering N glycosylation pathways in the baculovirus insect cell system, Curr. Opin. Biotech. 9:528 533.
Jenkins N, Curling EM (1994), Glycosylation of recombinant proteins: problems and prospects. Enzyme Microb. Technol. 16:354 364
Jenkins N, Parekh RB, James DC (1996), Getting the glycosylation right: implications for the biotechnology industry. Nat. Biotechnol. 14:975 981.
Kivirikko KI, Myllyla R, Pihlajaniemi T (1989), Protein hydroxylation: prolyl 4 hydroxylase, an enzyme with four cosubstrates and a multifunctional subunit. FASEB J. 3:1609 1617.
Kornfeld R, Kornfeld S (1985), Assembly of asparagine linked oligosaccharides, Annu. Rev. Biochem. 54:631 664.
Kulakosky PC, Shuler ML, Wood HA (1998), N Glycosylation of a baculovirus expressed recombinant glycoprotein in three insect cell lines, In Vitro Cell. Dev. Biol. 34:101 108.
Kunkel JP, Jan DC, Jamieson JC, Butler M (1998), Dissolved oxygen concentration in serum free continuous culture affects N linked glycosylation of a monoclonal antibody, J. Biotechnol. 62:55 71
Lando D, Peet DJ, Whelan DA, Gorman JJ, Whitelaw ML (2002), Asparagine hydroxylation of the HIF transactivation domain a hypoxic switch. Science 295:858 861.
Lazar AC, Kloczewiak MA, Mazsaroff I (2004), Matrix assisted laser desorption/ ionization mass spectrometry for the evaluation of the C terminal lysine distribu tion of a recombinant monoclonal antibody, Rapid Commun. Mass Spectrom. 18:239 244.
Leonard CK, Spellman MW, Riddle L, Harris RJ, Thomas JN, Gregory TJ (1990), Assignment of intrachain disulfide bonds and characterization of potential glyco sylation sites of the type 1 recombinant human immunodeficiency virus envelope glycoprotein (gp120) expressed in Chinese hamster ovary cells, J. Biol. Chem.

265:10373 10382.
Lopez M, Tetaert D, Juliant S, Gazon M, Cerutte M, Verbert A, Delannoy P (1999), O glycosylation potential of lepidopteran insect cell lines, Biochim. Biophys. Acta 1427:49 61.
Maras M, Saelens X, Laroy W, Piens K, Claeyssens M, Fiers W, Contreras R (1997), *In vitro* conversion of the carbohydrate moiety of fungal glycoproteins to mamma lian type oligosaccharides. Evidence for N acetylglucosaminyltransferase I accepting glycans from *Trichoderma reesei.* Eur. J. Biochem. 249:701 707.
Meynial Salles I, Combes D (1996), In vitro glycosylation of proteins: an enzymatic approach. J. Biotechnol. 46:1 14.
Nilsson MR, Driscoll M, Raleigh DP (2002), Low levels of asparagine deamidation can have a dramatic effect on aggregation of amyloidogenic peptides: implications for the study of amyloid formation. Protein Sci. 11:342 349.
Nyberg GB, Balcarcel RR, Follstad BD, Stephanopoulos G, Wang DI (1999), Meta bolic effects on recombinant interferon gamma glycosylation in continuous cul ture of Chinese hamster ovary cells, Biotechnol. Bioeng. 62:336 347.
Palacpac NQ, Yoshida S, Sakai H, Kimura Y, Fujiyama K (1999), Stable expression of human β 1,4 galactosyltransferase in plant cells modifies N linked glycosylation patterns, Proc. Natl Acad. Sci. U S A 96:4692 4697.
Parekh RB, Dwek RA, Edge CJ, Rademacher TW (1989), N glycosylation and the production of recombinant glycoproteins, Tibtech 7:117 122.
Pels Rijcken WR, Overdijk B, Van den Eijnden DH, Ferwerda W (1995), The effect of increasing nucleotide sugar concentrations on the incorporation of sugars into glycoconjugates in rat hepatocytes, Biochem. J. 305:865 870.
Petrescu AJ, Milac AL, Petrescu SM, Dwek RA, Wormald MR (2004), Statistical analysis of the protein environment of N glycosylation sites: implications for occupancy, structure, and folding, Glycobiology 14:103 114.
Raju TS, Briggs JB, Borge SM, Jones AJ (2000), Species specific variation in glycosyla tion of IgG: evidence for the species specific sialylation and branch specific galactosylation and importance for engineering recombinant glycoprotein thera peutics, Glycobiology 10:477 486.
Requena JR, Chao CC, Levine RL, Stadtman ER (2001), Glutamic and aminoadipic semialdehydes are the main carbonyl products of metal catalyzed oxidation of proteins. Proc. Natl Acad. Sci. U S A 98:69 74.
Rudd PM, Dwek RA (1997), Glycosylation: heterogeneity and the 3D structure of proteins, Crit. Rev. Biochem. Mol. Biol. 32:1 100.
Smales CM, Pepper DS, James DC (2002), Protein modification during anti viral heat treatment bioprocessing of factor VIII concentrates, factor IX concentrates, and model proteins in the presence of sucrose, Biotechnol. Bioeng. 77:37 48.
Spellman MW (1990), Carbohydrate characterization of recombinant glycoproteins of pharmaceutical interest, Anal. Chem. 62:1714 1722.
Stark NJ, Heath EC (1979), Glucose dependent glycosylation of secretory glycopro tein in mouse myeloma cells, Arch. Biochem. Biophys. 192:599 609.
Storring PL (1992), Assaying glycoprotein hormones the influence of glycosylation on immunoreactivity, Tibtech 10:427 432.
Sugiura T, Maruyama HB (1992), Factors influencing expression and post translational modification of recombinant protein C, J. Biotechnol. 22:353 360.
Thornalley PJ, Langborg A, Minhas HS (1999), Formation of glyoxal, methylglyoxal and 3 deoxyglucosone in the glycation of proteins by glucose, Biochem J. 344:109 116.
Van den Steen P, Rudd PM, Dwek RA, Opdenakker G (1998), Concepts and principles of O linked glycosylation, Crit. Rev. Biochem. Mol. Biol. 33:151 208.
Walmsley AR, Hooper NM (2003), Glycosylation efficiency of Asn Xaa Thr sequons is independent of distance from the C terminus in membrane dipeptidase, Glyco

biology 13:641 646
Xie L, Wang DI (1997), Integrated approaches to the design of media and feeding strategies for fed batch cultures of animal cells, Trends Biotechnol. 15:109 113.
Yan A, Lennarz WJ (2005), Unraveling the mechanism of protein N glycosylation, J. Biol. Chem. 280:3121 3124.
Yang M, Butler M (2000), Effects of ammonia on CHO cell growth, erythropoietin production, and glycosylation, Biotechnol. Bioeng. 68:370 380.

7 Mechanisms of cell proliferation and cell death in animal cell culture *in vitro*

Maíra Peixoto Pellegrini, Rodrigo Coelho Ventura Pinto, and Leda dos Reis Castilho

7.1 Introduction

The advances attained in the last decades in the knowledge of the biological fundamentals underlying animal cell culture have enabled significant improvements in cell culture processes *in vitro*. In this chapter, the mechanisms that determine cell proliferation and cell death are discussed. Aspects concerning the kinetics and the mathematical description of cell growth and cell death are dealt with in Chapter 8.

7.2 Cell proliferation mechanisms

Cell proliferation occurs through a sequence of coordinated events aimed at doubling the cellular material, so that cells can divide. This is known as the cell cycle and is the essential mechanism governing reproduction of all eukaryotic cells. The basic function of the cell cycle is the error-free duplication of the genetic material (DNA – deoxyribonucleic acid) contained in the chromosomes, followed by a precise segregation of the copies into two genetically identical daughter cells. During the cell cycle, total proteins and RNA are duplicated and two cells of similar size with respect to volume and mass are formed (Mitchison, 2003).

The duplication process defines two important phases of the cell cycle (Griffiths, 1984; Alberts *et al*., 2002). In the S phase (synthesis), which in mammalian cells lasts 10–12 hours (representing half of the cell cycle), DNA duplication occurs. In the M phase (mitosis), which lasts less than 1 hour in mammalian cells, segregation of chromosomes and cell division take place. This phase is divided into six stages: prophase, prometaphase, metaphase, anaphase, telophase, and cytokinesis (*Figure 7.1*). In the initial stages, the duplicated DNA strands are condensed into more compact chromosomes, which are necessary for segregation. Subsequently, the chromosome copies bind to the mitotic spindle, which consists of a bundle of microtubules. Its function is to segregate chromosomes during cell

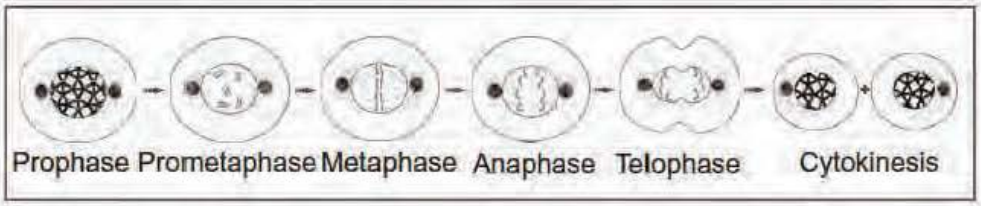


Figure 7.1

The nuclear division process during the M phase lasts less than an hour. The M phase can be divided into six distinct stages, according to the progression of nuclear division (prophase, prometaphase, metaphase, anaphase, telophase, and cytokinesis) (adapted from Alberts *et al.*, 2002).

division. The chromosomes are aligned and move to opposite poles of the cell, that is, to the opposite ends of the mitotic spindle. At this stage they are less compact and they form two new nuclei. The final stage of this process, known as cytokinesis, consists of the complete division into two cells, each containing part of the original cytoplasm.

Cell division also requires duplication of the protein mass and of organelles, which takes longer to be accomplished than DNA replication. Thus, for the cell to be able to grow and duplicate its contents, there are two additional phases in the cell cycle: the G_1 phase (between the M and the S phase), and the G_2 phase (between the S-phase and mitosis). The cell cycle is divided into four sequential phases: G_1, S, G_2, and M (*Figure 7.2*). The part of the cycle that consists of phases G_1, S, and G_2 is designated the interphase. In a typical proliferating mammalian cell, the interphase can

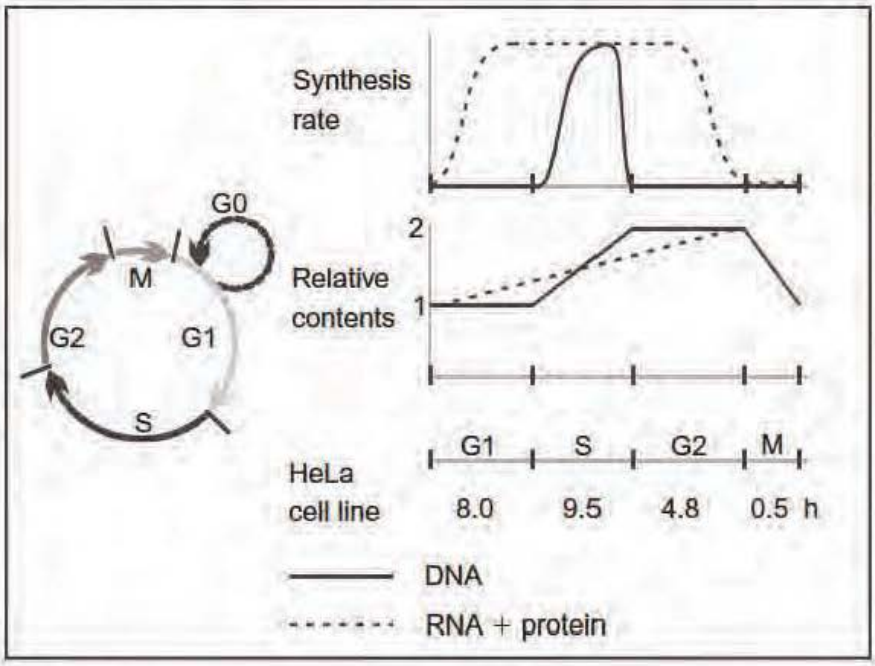


Figure 7.2

Cell cycle phases versus synthesis rate and concentration of macromolecules in the cell, as well as average duration of cell cycle phases for the tumor HeLa cell line (adapted from Griffiths, 1984).

last 23 hours and mitosis 1 hour, resulting in an average doubling time (t_d) of 24 hours (Griffiths, 1984; Alberts *et al*., 2002). Depending on the environmental conditions and the characteristics of the cell (e.g. tumor cells), the doubling time can vary.

The two G phases represent mainly time intervals when the cell can grow, but important regulatory phenomena also occur during G_1 and G_2. In these phases, the cell monitors the intra- and extracellular environment to ensure that there are adequate conditions and that the cell itself is prepared to move forward to the next phase. G_1 has a rather variable duration, which depends on extracellular conditions and external signals. If the conditions are not suitable, the cell delays its progression through G_1 and may even enter a quiescent phase designated G_0, where it can remain for an indefinite period of time until restarting cell proliferation. If the extracellular environment is favorable and there are growth factors available, a cell in G_1 and G_0 proceeds up to a point where it cannot be detained any more, known as point of restriction. After transposing this point, the cell is fated to replicate its DNA, even if the environment becomes adverse for its development (Griffiths, 1984; Alberts *et al*., 2002).

The cell division process is governed by a complex regulatory mechanism, which comprises signals from different sources that indicate when division is necessary and appropriate. These signals originate from both the extracellular and intracellular environment. The signals consist of growth factors and hormones, which help cells to determine when it is necessary for them to divide, if the necessary nutrients are available, and if there are any signals contrary to the start of the reproductive cycle. The signals relating to the intracellular verification points, at the end of phases G_1 and G_2, are necessary to coordinate in time and space the different cell cycle events, ensuring that a complete sequence of events occurs before a new sequence begins. If DNA damage or poor assembly of the mitotic spindle occurs, the cycle progression is interrupted until repair measures are successfully accomplished (Fussenegger and Bailey, 1998; Li and Zou, 2005).

The intracellular control of the cell cycle is carried out by a family of proteins known as cyclin-dependent kinases (CDKs). The activity of these kinases varies according to the progression of the cell in the cycle. Alterations in the activity of these proteins result in cyclic changes in phosphorylation of intracellular proteins, which indicate or regulate the main cell cycle events. These cyclic changes that may occur in the activity of CDKs are in turn controlled by other proteins, known as cyclins. As the name suggests, CDKs depend on cyclins for appropriate activity, since they are only active when bound to a specific cyclin. Unlike the level of CDKs, which is approximately constant, cyclins present cycles of synthesis and degradation, which result in a cyclic formation and activation of cyclin–CDK complexes. This activation, in turn, activates the cell cycle events of animal cells (Alberts *et al*., 2002). Other important proteins and intracellular factors also participate in the regulation of cell proliferation and cell death. The protein known as p53 is activated upon DNA damage during replication, interrupting cell cycle progression and activating cell death or senescence mechanisms (Harris and Levine, 2005). The family of polo-like kinases (PLKs) mediates the control of the checkpoints that monitor events such as replication and segregation (Xie *et al*., 2005).

When cells from normal mammalian tissues are cultivated *in vitro* under adequate conditions, they usually stop dividing after a given number of cycles, and this is called replicative cellular senescence. Hayflick and Moorhead (1961) first recognized that cells have a limited lifespan *in vitro*. They worked with human fibroblasts (WI-38 cell line) and observed that these cells died after about 50 population doublings (Hayflick and Moorhead, 1961; Hayflick, 1965). Senescence is related to changes in the structure of telomeres, which are repetitive DNA sequences associated with proteins at the tips of the chromosomes. When cell division occurs, the telomere sequences are not replicated in the same way as the rest of the DNA. These sequences are synthesized by the telomerase enzyme, which is also responsible for the addition of proteins that protect the chromosome ends. In the germ line, during early development, and in highly proliferative organs, telomere shortening and elongation is balanced. However, when cells become fully differentiated or mature, the shortening process becomes dominant due to telomerase repression (Bekaert *et al*., 2004). Thus, the telomeres become shorter and their protective proteins deteriorate with time. With their ends exposed, the chromosomes become prone to damage, and this may cause an arrest of the cell cycle or induce cell death mechanisms (Alberts *et al*., 2002). Normal rodent cells possess active telomerase throughout their proliferative lifespan, and this explains why they are so readily immortalized and transformed in culture compared with their human counterparts. Telomerase is present in 90% of human cancer cells, and therefore it represents an attractive target for developing new anticancer drugs (Newbold, 2002).

Although the use of immortalized cell lines has allowed the establishment of different cell culture technologies for the large-scale production of biopharmaceuticals, an enhanced capacity for proliferation may become disadvantageous. In batch cultures, for instance, the aim is to reach high cell concentrations, but this results in exhaustion of essential nutrients and accumulation of toxic metabolites, leading to sub-optimal nutritional conditions that lead to cell death and to the end of the production process (Fussenegger and Bailey, 1999). However, in many cases, the synthesis of a product is not associated with cell growth and maximum productivity is reached under conditions in which cell proliferation is decreased, but high viabilities are maintained. Therefore, some cultivation processes are based on operational strategies that allow cells to remain viable, but in a non-proliferative state, so as to prolong the productive phase and to increase the productivity of the process (Suzuki and Ollis, 1989, 1990; Fussenegger and Bailey, 1999).

By these strategies cell proliferation may be controlled by adding chemical additives that arrest the cell cycle, usually in the G_1 phase, increasing specific productivity. However, concomitantly undesirable effects such as cytotoxicity may be observed, which result in a decrease in cell viability and in the impossibility of maintaining the culture in a nonproliferative state for long periods of time (Suzuki and Ollis, 1990; Al-Rubeai *et al*., 1992). Deprivation of specific nutrients and growth factors can also stop cell proliferation, but in this case cell viability decreases and programmed cell death – apoptosis – is activated (Mercille and Massie, 1994a, 1994b; Singh *et al*., 1994; Fussenegger and Bailey,

1999). Currently, much research on the biochemical control of cell cultures based on preventing the cell death mechanisms, to avoid cell death instead of inhibiting cell growth, is being carried out with the aim of prolonging the productive period of a cell culture process (Al-Rubeai and Singh, 1998; Fussenegger *et al*., 1999; Fussenegger and Bailey, 1999). This biochemical control is based on the knowledge and manipulation of the molecular basis of cell death, as discussed in detail in Section 7.6 of this chapter.

7.3 Cell death mechanisms: apoptosis and necrosis

Cell death occurs by two different mechanisms: necrosis or apoptosis. The term ''apoptosis'' was first used in 1972 by Kerr, Wyllie, and Currie to describe a form of cell death that differed from cell death by necrosis in that no damage to the cell membrane and no inflammatory responses were observed (Kerr *et al*., 1972). Apoptosis was later recognized as an important physiological process during the life of all multicellular organisms, where cells are regularly lost. These cells are removed when they are no longer needed or when they represent a danger. This allows the tissue to function normally. The perfect balance between proliferation and cell death maintains homeostasis. Examples of apoptosis during the life of an organism are: tissue renovation, positive and negative selection of lymphocytes, elimination of cells during embryogenesis (such as the formation of limbs), and development of the nervous system of vertebrates. An appropriate control of apoptosis is crucial for higher eukaryotes, and any failure in its regulation can lead to diseases. The lack of a mechanism for apoptosis in tumor cells permits them to survive and, thus, contributes to cancer development. It can also be responsible for autoimmune diseases. Excessive or inappropriate activation of apoptosis may cause degenerative diseases, such as osteoporosis and Alzheimer's or Parkinson's diseases.

Necrosis is considered an accidental death, and thus a non-physiological process, which is characterized by changes in morphology and in mitochondrial function, as well as by the inability of the plasma membrane to regulate osmotic pressure inside the cell. Initially, there is an increase in the cytoplasmic volume due to the influx of liquid into the cell (*Figure 7.3*). This is followed by membrane and organelle rupture, leading to the release of lysosomes and cytoplasmic material, and culminating in random nuclear fragmentation (Al-Rubeai, 1998). The damage to the plasma membrane includes alterations in the ion transport system, followed by an increase in the permeability of the membrane and cell swelling (Buja *et al*., 1993).

Apoptosis in turn is characterized by a series of morphological alterations, in an autodestruction process that results in the formation of vesicles containing cell material, known as apoptotic bodies, which, *in vivo*, are subsequently phagocytized by specialized cells. This series of events is initiated by a given stimulus that elicits a cascade of intracellular signals, activating enzymes (caspases) that will mediate the morphological and physiological changes experienced by the cell. In general, apoptosis differs from necrosis by the absence of cytoplasmic material leakage, by the

decrease in cell size, by a controlled and uniform DNA fragmentation, and by specific changes in the chromatin, which becomes highly condensed and can appear concentric with the nuclear membrane. The only alteration that may be observed in the organelles is a swelling of the endoplasmic reticulum (ER) (McCarthy, 2002). However, during cultivation processes *in vitro*, apoptosis ends up in a stage called secondary necrosis (*Figure 7.3*; see color section), where, due to the absence of specialized phagocytic cells, the apoptotic cells and apoptotic bodies release their contents into the extracellular medium, as occurs in necrosis (Al-Rubeai, 1998).

7.4 Influence of environmental conditions on the induction of cell death

During *in vitro* cultivation of animal cells, any change relative to the optimal environmental conditions may result in a rapid decrease in cell viability. This phenomenon is more frequently observed in cell lines that exhibit high susceptibility to apoptosis (Singh and Al-Rubeai, 1998). Any agent or condition that alters the cell metabolism may activate programmed cell death. Cells exposed to high stress levels due, for instance, to the presence of a high concentration of toxins, to large changes in pH, or to high agitation rates, generally die by necrosis, because there is no time for the cell to generate a response, that is, cell death is instantaneous. At intermediate stress levels, the cell can be damaged without instantaneously dying, having time to activate its programmed death mechanism. Under low levels of environmental stress, the cells may produce heat-shock proteins (HSPs), which allow their survival until the stimulus is eliminated. However, once a threshold level of resistance is surpassed, survival becomes impossible and the cells activate their apoptotic pathways (Cotter and Al-Rubeai, 1995).

Cells in culture are usually exposed to variations in environmental conditions and, depending upon these conditions, cells can survive or die. In biotechnological processes, the usual aim is to maintain cells alive and productive. There are a large number of factors that influence the quality of a cell culture, including an appropriate supply of nutrients, pH, shear stress, accumulation of metabolites, and oxygen availability. Studies have already shown that several alterations in culture conditions, such as the lack of nutrients or serum, increase in osmotic pressure, and maintaining cells in suspension culture, may induce apoptotic mechanisms. The critical factors determining apoptosis vary between cell lines (Singh *et al*., 1994; Zanghi *et al*., 1999; Kim and Lee, 2002).

7.4.1 Depletion of nutrients and growth factors

Cells survive and proliferate according to the addition of appropriate amounts of nutrients and growth factors, which are provided by the culture media. When these requirements are not met, cells die. The availability of nutrients and growth factors is considered the most significant factor influencing the biological activity of cells. The lack or depletion

of these substances can not only lead to arrest of proliferation, but also to cell death by apoptosis.

In the cells, control of proliferation and apoptosis is carried out by an integrated signaling mechanism that activates or avoids one of these processes and also influences other aspects of cell activity. For instance, the presence of mitotic agents that induce proliferation, and survival factors that inhibit apoptosis, are necessary for maintaining cell viability (Al-Rubeai, 1998). It is important, however, to distinguish between mitogens and survival factors. Insulin-like growth factor 1 (IGF-1) is a potent survival factor, but a poor mitogen. Many cytokines are good mitogens, but poor survival factors, since they induce cell division but do not avoid apoptosis. In this way, induction of cell growth and apoptosis inhibition become independent events. Macrophage-colony stimulating factor (M-CSF), for instance, promotes cell survival when present at low concentrations, but promotes proliferation at high concentrations. Thus, the correct balance of growth and survival factors is an important consideration for maintaining high cell viability in culture (Cotter and Al-Rubeai, 1995).

Animal serum, especially serum from bovine fetuses, plays an important role in the growth and survival of animal cells in culture. Serum contains growth factors, proteins, and other nutrients. The removal of serum has been associated with apoptotic induction in different cells, including hybridomas and Chinese hamster ovary (CHO) (Zanghi *et al*., 1999) and plasmacytomas (Singh *et al*., 1994). The mechanism by which the removal of growth factors, such as those contained in serum, induces apoptosis seems to involve the expression of the proto-oncogene *c-myc* (Al-Rubeai, 1998). In CHO cells, apoptosis is induced by nutrient depletion due to accelerated nutrient consumption in the absence of serum. On the other hand, when serum or plasma is present at high concentrations, death by apoptosis is also induced, but this phenomenon can be avoided by adding supplements containing thiol groups, such as L-cysteine or L-tryptophan (Al-Rubeai, 1998).

When cells are deprived of an energy source such as glucose or glutamine, apoptosis can be induced. The effects of lack of glutamine on apoptosis induction have been observed in hematopoietic cells, such as hybridomas and plasmacytomas (Mercille and Massie, 1994a; Singh *et al*., 1994). Sanfeliu and Stephanopoulos (1999) demonstrated that the lack of glutamine is the major factor activating apoptosis in a CHO cell line producing interferon-gamma, and that this effect could be attenuated by the expression of the gene Bcl-2, which codes for an anti-apoptotic protein that participates in the signaling pathway that is triggered in response to apoptotic stimuli. The influence of glucose and glutamine on the apoptotic pathways is not yet completely understood, but the concentrations of both compounds regulate cell growth and the catabolic pathways. Their depletion can block DNA synthesis and arrest the culture in the G_1 phase, causing apoptosis. However, this does not explain why cells in phases S, G_2, and M also undergo apoptosis. It is suggested that apoptosis can be induced when the energy required for synthesis of the ATP, needed for the duplication of cellular material, is decreased to a critical level, resulting in the activation of regulatory proteins that promote apoptosis (Al-Rubeai, 1998).

The relationship between apoptosis induction and glutamine depletion in batch cultures of hybridomas led to studies suggesting that apoptosis is activated upon depletion of any amino acid. Although the lack of essential amino acids is critical, the depletion of any amino acid can induce apoptosis, since the synthesis of molecules involved in the regulation of cell death is blocked (Simpson *et al*., 1998). There are two probable explanations for this. According to one of them, the decrease in the intracellular concentration of amino acids can lead to a depletion of tRNA molecules, blocking translation (protein synthesis). According to the second theory, transcription and translation can be impaired by the decrease in the intracellular ATP levels. Although not all amino acids contribute to the energetic requirements of the cells, the absence of any amino acid results in the induction of apoptosis. Experiments with murine hepatocytes showed that protein synthesis is inhibited at the level of the initiation of the peptide chain. This indicates that the role of amino acids as protein precursors could be one of the most significant factors inducing death by apoptosis (Singh and Al-Rubeai, 1998).

7.4.2 Oxygen limitation

The apparent involvement of free radicals in apoptotic processes has led to studies of the effects of dissolved oxygen tension on animal cells in culture. Hydrogen peroxide was demonstrated to be a factor inducing apoptosis, whereas antioxidants such as catalase and superoxide dismutase protect cells. Formerly, it was believed that the Bcl-2 protein acted as an antioxidant, but today it is known that anoxic conditions can induce apoptosis (Mercille and Massie, 1994a) and that Bcl-2 expression presents an anti-apoptotic action also under limiting conditions of oxygen. These studies are very important for large-scale cultivation processes and for processes where oxygen may be limiting, since the supply of oxygen is a key factor that limits the viable cell concentration attained in these culture systems. The effects of Bcl-2 protection under anoxic conditions have already been demonstrated for different cell lines. For cells derived from Burkitt's lymphoma transfected with the Bcl-2 gene, the resistance to anoxic conditions increased, and cell viability could be maintained at high levels for a longer period than with control cells (Singh *et al*., 1997). In the case of a hybridoma, the expression of this gene also conferred a higher viability and integrity of the cells when exposed to anaerobic conditions (Simpson *et al*., 1997).

7.4.3 Susceptibility to shear stress

Animal cells do not have a cell wall and are, thus, highly susceptible to the effects of shear stress. Cells respond to hydrodynamic stress within minutes, altering their metabolism and the gene expression pattern (Nollert *et al*., 1991). Under sub-lethal levels of shear stress, there is initially an increase in passive transmembrane transport, simultaneously with damage to surface receptors. The plasma membrane is generally the main site for shear damage, and it may lose its capacity to mediate the transport of ions and molecules, so that the cell loses its viability. It has been demonstrated

that the susceptibility of some cell lines to the effects of hydrodynamic stress is related to cell size and cell cycle, with cells in S and G_2 phases more prone to damage and smaller cells more resistant to injury. It has also been observed that cell damage and the dominating death mechanism (necrosis or apoptosis) depend on the intensity and exposure time to shear stress (Al-Rubeai *et al*., 1995a, 1995b). Exposing hybridoma cells to moderate agitation levels resulted in a decrease of viability and an increase in cell death by apoptosis (Al-Rubeai *et al*., 1995b), whereas extreme shear levels led to cell death by necrosis, with complete cell fragmentation.

The hydrodynamic environment in a stirred-tank bioreactor can be stressful for animal cells. This arises from the high energy introduced into the system to maintain homogeneity and cells in suspension. Furthermore, gas bubbling results in cell damage, especially at the gas–liquid interface near the headspace of the bioreactor. While knowledge of the effects of hydrodynamic conditions has improved bioreactor design and operation strategies, so as to decrease hydrodynamic stress, a compromise still exists concerning the need for efficient agitation and aeration and the susceptibility of cells to shear. Certainly, if apoptosis is induced by hydrodynamic stress, then the suppression of this death mechanism, e.g. by molecular strategies, would allow enhanced agitation and aeration, improving mass transfer with a decreased effect on cell viability (Singh and Al-Rubeai, 1998).

7.4.4 Osmolality

Culture medium osmolality has a large influence on animal cell culture. Hyperosmotic conditions, achieved by using salts, CO_2, or concentrated media, has been shown as a low-cost method to increase specific productivity of cells. Hybridoma cultures, for instance, show improved productivity with an increase in osmolality (Oh *et al*., 1993). However, this method is not very popular due to the negative effects on cell growth. The drop in cell growth under hyperosmotic conditions occurs, probably, due to cell death by apoptosis. Thus, expression of anti-apoptotic genes, such as Bcl-2, could allow the use of hyperosmotic conditions to simultaneously limit cell death and increase cell productivity (Kim and Lee, 2002).

7.5 Methods of detection of cell death by apoptosis

Currently, there are many methods available for determining cell death by apoptosis in cell cultures and tissues. These methods are based essentially on changes that occur in apoptotic cells. During apoptosis, several phenomena can be observed, such as DNA fragmentation, chromatin condensation, nuclear fragmentation, cytoplasm acidification, cytochrome *c* release from the mitochondria, exposure of intracellular phospholipids and the activation and breakdown of proteins. These apoptotic phenomena can be detected by direct or indirect methods, on cell populations or on individual cells that are representative of a population. The main principles used by the different detection methods are:

(i) DNA fragmentation;
(ii) morphological changes;
(iii) changes in membrane asymmetry;
(iv) activation of apoptotic proteins;
(v) release of cytochrome *c* in the cytoplasm.

7.5.1 DNA fragmentation

During controlled cell death, the DNA is initially cleaved into 300–500 kb fragments (Brown *et al*., 1993). Later on, these fragments are decreased to multiples of 180–200 bp, which is the distance between histone proteins, around which the condensed chromatin is wrapped. This phenomenon occurs simultaneously with morphological changes, such as chromatin condensation. The resulting fragments are known as LMW-DNA (low molar mass DNA) fragments, which are able to pass through pores of the nuclear membrane and reach the cytoplasm, and HMW-DNA (high molar mass DNA) fragments, which remain in the nucleus. The main enzyme involved in this process is a caspase-activated DNAse (CAD) (Nagata, 2000).

Agarose gel electrophoresis

The DNA fragments can be visualized in electrophoretic runs in agarose gels showing a distinct band pattern (DNA laddering). The sizes of the bands are multiples of the smallest nucleosomal fragments. Unlike those of apoptosis, the DNA fragments arising from necrosis are of irregular size, resulting in a smear in the sample lane. Although the gel electrophoresis pattern is a biochemical indication of apoptosis, in some cases the classic morphological features of apoptosis may be noted without DNA fragmentation (Al-Rubeai, 1998; Nagata, 2000).

TUNEL

TUNEL stands for terminal deoxynucleotidyl transferase x-dUTP nick end labeling. This assay is based on the detection of DNA fragments marked by an enzyme that incorporates modified nucleotides to the 3′-OH ends of the fragments, which can be then specifically detected. The enzyme is a deoxynucleotidyl transferase, which can act in absence of a complementary strand. Among the nucleotides, there is one specifically marked with a fluorochrome, an enzyme, or an antigen. This allows different methods of detection.

When the deoxynucleotide is associated with a fluorochrome, the cells can be observed under a fluorescence microscope, whereby apoptotic cells present an intense fluorescence and, in advanced stages, nuclear fragmentation can be visualized. For a quantitative analysis, either a hemocytometer in an optical microscope or a flow cytometer can be used (Tinto *et al*., 2002). Peroxidase-marked deoxynucleotides can be quantified by chromogenic tests that use the enzyme substrate. Indirect methods use antigens linked to the nucleotide and recognized by labeled antibodies. However,

this is a less sensitive technique, which is used mainly in pre-fixed histological samples.

In the TUNEL test, it is necessary to permeabilize the cells to introduce the enzyme and the deoxynucleotides, but the permeabilization is carried out after weak fixation with formaldehyde, so that low molar mass fragments are not lost. Permeabilization is carried out in an ice bath, followed by labeling with the reaction solution.

Propidium iodide, ethidium bromide, and DAPI

These methods use fluorescent labels, such as propidium iodide, ethidium bromide, or DAPI (4′,6′-diamidino-2-phenylindole), which are incorporated into the DNA, allowing chromatin condensation and nuclear fragmentation to be visualized under a microscope with the appropriate fluorescence filters. To allow fluorochromes to enter the cells and reach the nucleus, the cells need to be prepermeabilized, for example, with 70% ethanol at −20°C. LMW-DNA fragments may be lost by the permeabilization, decreasing the amount of DNA inside the cells. The lower nucleic acid concentration results in a lower fluorescence intensity in apoptotic cells, which can be detected by fluorescence microscopy or flow cytometry (Calle *et al*., 2001).

7.5.2 Morphological changes

Several methods are based on microscopy observations to detect morphological changes in the cells, mainly chromatin condensation. Different markers can be used to differentiate between the stages of apoptosis.

To distinguish apoptotic from normal and necrotic cells, fluorescent dyes can be used to form complexes with DNA, such as acridine orange, ethidium bromide, or propidium iodide (Mercille and Massie, 1994b). Fluorescence microscopy following cell labeling clearly reveals chromatin condensation and fragmentation in apoptotic cells. By combining the use of fluorescent dyes that are able to label the chromatin in intact cells (e.g. acridine orange) with those that just label the chromatin in cells with a damaged membrane (e.g. ethidium bromide and propidium iodide), it is possible to identify different levels of cell viability (Mercille and Massie, 1994a).

Acridine orange is able to penetrate cells independently of the integrity of the cell membrane. It intercalates the bases in double strands of DNA and emits green fluorescent light. It can also form complexes with RNA and single-stranded DNA, but in this case it does not intercalate the bases and emits red fluorescent light. Thus, a viable cell can be visualized with a green nucleus and, possibly, reddish spots in the cytoplasm.

Ethidium bromide is not able to permeate the plasma membrane, and only penetrates non-viable cells that have lost the selective permeability of the membrane. It can intercalate the bases of double-stranded DNA molecules, emitting an orange fluorescent light. Ethidium bromide also forms weak complexes with RNA, resulting in red fluorescence. Thus, non-viable cells will present a strong orange fluorescence, since ethidium

bromide labeling overlays acridine orange fluorescence. If there is still cytoplasmic material inside the cell, it will be labeled dark red.

As shown in *Figure 7.3* (see color section), the morphologies observed in a sample incubated with acridine orange and ethidium bromide allow the identification of four levels of cell viability, as described below:

(i) Viable nonapoptotic (VNA) cells show the nucleus uniformly colored green, with no chromatin condensation. In this cell population, only acridine orange is able to penetrate the cells and bind to the DNA.
(ii) Early apoptotic cells, also called viable apoptotic (VA), show brilliant green granules in the nuclear region. These granules correspond to the condensed and fragmented chromatin, which means that the cells have already initiated the apoptotic process, but still keep the selective permeability of the membrane. In this phase, the formation of apoptotic bodies containing material that will be expelled by the cells can be observed.
(iii) Late apoptotic cells, also known as non-viable apoptotic (NVA), are colored reddish-orange, with granules in the nuclear region showing brilliant orange fluorescence. The cells have lost membrane integrity and have become permeable to ethidium bromide. Another characteristic of this phase is the decrease in cell size, due to the loss of cell material as a result of elimination of apoptotic bodies.
(iv) Necrotic cells are totally colored orange, indicating loss of selective permeability of the membrane. These cells do not show any condensation or fragmentation of the chromatin, and present no decrease in size. On the contrary, there is a tendency for an increase in cell volume. No apoptotic bodies are formed. This stage is very short, and cell lysis occurs within a short time.

7.5.3 Membrane asymmetry

Translocation of the phosphatidylserine from the inner to the outer leaflet of the plasma membrane is an initial event related to the apoptotic process and possibly serves as a signal for the removal of apoptotic bodies by phagocytic cells (Martin *et al*., 1995). The exposure of this phospholipid has been largely used as a specific apoptosis marker.

Membrane asymmetry changes can be detected by flow cytometry using a fluorescent marker (e.g. fluorescein, FITC) conjugated to annexin V, a protein that has high affinity for phosphatidylserine. When using a fluorescence microscope, this technique can be quantitative if a hemocytometer is used.

Unfortunately, annexin V also binds to phosphatidylserine residues in the inner leaflet of the plasma membrane of necrotic cells due to the loss of integrity of the membrane. However, the use of propidium iodide as a counter-marker allows viable, apoptotic, and necrotic cells to be distinguished (Pläsier *et al*., 1999).

7.5.4 Apoptotic proteins

There are a large number of genes related to apoptosis, and the corresponding proteins modulate the cell death process. Tests that detect these proteins or their activities are largely used to detect apoptosis in cell culture. This group of apoptotic proteins is very large and, theoretically, any key protein participating in apoptotic pathways, such as p53 protein and caspases 3 and 7, can be used to detect apoptosis (Kim and Lee, 2002; Arden and Betenbaugh, 2004).

7.5.5 Cytochrome *c* release

Alterations of the mitochondrial membrane may precede those that occur in the nucleus. These changes can involve both the inner and the outer membrane, leading to a dissipation of the transmembrane potential and/or to the release of intermembrane proteins through the outer membrane. The main group of proteins responsible for mitochondrial alterations consist of the proteins known as Bax, which form pores in the outer membrane, causing the release of cytochrome *c* to the cytoplasm (Loeffler and Kroemer, 2000; Arden and Betenbaugh, 2004). Western blot techniques can be used to specifically detect the presence of cytochrome *c* in the cytoplasm of apoptotic cells. However, complex purification protocols are required, and there is the possibility of incomplete separation of mitochondria from the cytoplasm; therefore, this technique is not very popular.

7.6 Apoptosis suppression by molecular techniques

7.6.1 Molecular basis of apoptotic cell death

Much of the current knowledge of the genetic regulation of apoptosis began with genetic studies of the nematode *Caenorhabditis elegans*, in which 131 of 1090 somatic cells formed during adult development die by apoptosis. This cell death is regulated by a group of approximately 13 genes (Horvitz *et al*., 1994). The apoptotic mechanism identified in *C. elegans* seems to be highly evolutionarily conserved in vertebrates and invertebrates, since homolog genes were also discovered in mammals, although in a much greater amount.

The events involved in apoptosis can be divided into four phases: initiation, signaling, effector, and degradation (Mastrangelo and Betenbaugh, 1998). During the initiation phase, the cell is exposed to insults that trigger the apoptotic cascade. The main insults found in cell cultures are: nutrient depletion (such as glucose, glutamine, and other amino acids), deprivation of growth and survival factors (e.g. factors in fetal bovine serum), accumulation of toxic metabolites (ammonia and lactate), oxygen concentration (hypoxia and hyperoxia), hydrodynamic stress, loss of cell adhesion (e.g. during suspension adaptation), viral infection (e.g. when viral vectors are used for recombinant DNA transfer), and protein overexpression. Once apoptosis has been triggered (initiation), the message is transmitted through the signaling cascade during the signaling phase. This

stage of apoptosis includes several parallel pathways that converge to a few or even a single pathway of the effector phase. Each insult probably activates a different signaling cascade pathway, finally leading to the effector phase, which involves the activation of caspases, the proteases responsible for cellular degradation (Dickson, 1998). During this last phase, which is irreversible, it is possible to observe the specific morphological changes of apoptosis, such as chromatin condensation, nuclear fragmentation, cellular shrinkage, blebbing of plasma membrane, and formation of apoptotic bodies. Different cell lines respond to different insults with varying intensities, possibly as a result of activation of different apoptotic pathways (Singh and Al-Rubeai, 1998).

The caspase family

The characteristic morphological features of apoptosis are due to the direct or indirect action of caspases, a highly conserved protease group (Alnemri *et al*., 1996). Caspases are cysteine-aspartate proteases, members of a cysteine-protease family that specifically cleave their substrates after aspartic acid residues. To date, at least 14 mammalian caspases have been identified. Although they share a common structure, they can be divided into two functional groups: those that participate in the apoptosis process (caspases 2, 3, 6, 7, 8, 9, 10, and 12) and those that are responsible for cytokine processing during immune response and are involved in the inflammatory process (caspases 1, 4, 5, and 11) (Alnemri *et al*., 1996). However, due to the lack of functional information on some of the caspases (e.g. caspases 2, 4, 5, 7, 11, 12, and 13), the exact division between the two groups is still ambiguous.

Caspases are expressed as zymogens or procaspases and require a proteolytic cleavage at specific aspartic acid residues to become active. These cleavages generate four fragments: the N-terminal prodomain (2–25 kDa), the large subunit (17–21 kDa), the C-terminal small subunit (10–13 kDa), and a small linker between large and small subunits (*Figure 7.4*). Some caspases do not possess the small linker. An active caspase consists of a heterotetramer containing two small and two large subunits, which are derived from two procaspase molecules (Walker *et al*., 1994). The N-terminal prodomain and the linker (if present) are removed from the mature and active caspase. The observation that caspases, to become active, undergo the same process as their substrates (they are cleaved at aspartic acid residues) suggests a caspase proteolytic cascade, that is, an active caspase can subsequently cleave and activate other procaspases (Thornberry *et al*., 1997).

Caspases involved in the apoptotic process can be subclassified as initiator or effector caspases, depending on the structure of their prodomain. This also reflects their different roles in the apoptotic cascade (Earnshaw *et al*., 1999).

Initiator caspases (caspases 2, 8, 9, 10, and 12) possess a functional prodomain. This prodomain is large and contains interaction domains (structural motifs) where adaptor proteins, like Apaf-1 and FADD, can bind. The interaction with adaptor proteins is important for the caspase activation process. There are two types of interaction domains: DED

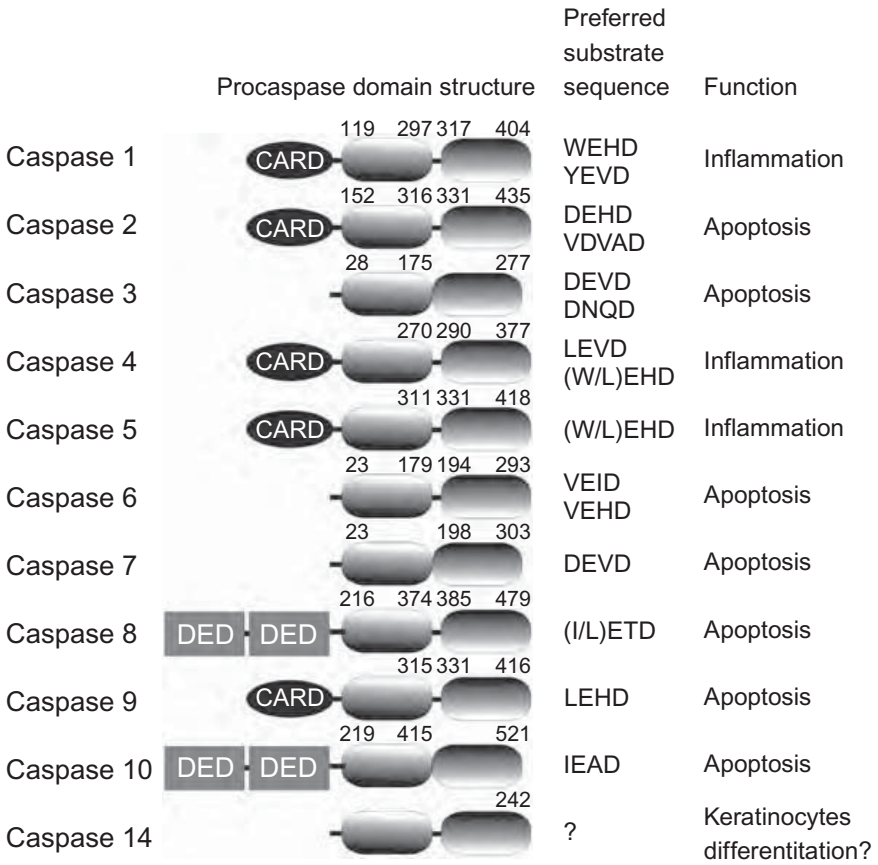


Figure 7.4

Caspase family. This scheme illustrates the domain structures, internal cleavage sites, preferred peptide substrate sequences, and biological function of caspases. Each procaspase consists of a large and small domain and may also possess DED (death effector domain) and CARD (caspase recruitment domain) (adapted from Hill *et al.*, 2003).

(death effector domain) and CARD (caspase recruitment domain) (Muzio *et al*., 1996; Hofmann *et al*., 1997). The contact with CARD domains is based on electrostatic interactions, while the contact with DEDs is based on hydrophobic interactions. Caspases 2, 9, and 12 have CARDs and caspases 8 and 10 have DEDs. Generally, initiator caspases act upstream of the effector caspases and are responsible for their activation (Thornberry *et al*., 1997; Earnshaw *et al*., 1999).

The main activation mechanism of initiator caspases is autoproteolytic, which is dependent on procaspase aggregation. Caspases possess low intrinsic enzymatic activity when in a zymogen form. Their catalytic activity increases when they are cleaved and assembled into tetramers (Yamin *et al*., 1996). It has been observed that procaspase aggregation or oligomerization facilitates intermolecular proteolysis, which results in autocatalysis (Srinivasula *et al*., 1998; Yang *et al*., 1998). Adaptor proteins recruit and bring together initiator caspases into activating complexes, which allows the autoactivation.

Effector caspases (caspases 3, 6, and 7) may possess a small inactive prodomain or may lack it completely, since they do not need to form

aggregates to be active. After being activated, generally by initiator caspases, the effector caspases are responsible for the death signal amplification, for example, caspase 9 activates others caspases, like caspase 3, which in turn cleaves and activates more caspase 9, thus amplifying the apoptotic signal (Slee *et al*., 1999).

Effector caspases are activated by a transactivation mechanism, which is characterized by the catalytic action of a mature caspase on a procaspase (Thornberry *et al*., 1997; Earnshaw *et al*., 1999; Slee *et al*., 1999). Nevertheless, their activation can also occur by the action of other proteases. Granzyme B, a serine-protease, also has proteolytic specificity for aspartic acid residues. It is able to cleave and directly activate caspase 3 (Darmon *et al*., 1995). Cathepsin B, a lysosomal protease, cleaves and activates procaspase 11 (Schotte *et al*., 1998).

Effector caspases also interact with other molecules besides caspases. These caspases interact and cleave key regulatory and structural proteins (Earnshaw *et al*., 1999) that can be directly inactivated, directly activated, or can modulate the function of other proteins as a result of cleavage. The main substrates directly inactivated are structural proteins, which lose their function, like cytoskeleton proteins (actin, gelsolin, α-foldrin); components of gap junctions (β-cathenin, plakoglobin), and nuclear proteins (lamin A and B); proteins involved in metabolism and DNA repair (DNA topoisomerase II, PARP); signaling proteins, like transcriptional activators (NFκB) and kinases (Akt, FAK); and antiapoptotic proteins (Bcl-2, Bcl-X_L). The cleavage of cytoskeleton and gap junction proteins results in cells becoming spherical and detaching from the surface and from neighbor cells. The cleavage of lamin A and B contributes to the break up of the nucleus into vesicles. Examples of proteins activated after cleavage are the caspases themselves, proapoptotic proteins, like Bid and Bax, and kinases (PAK2, MEKK1). These two kinases, when activated, are capable of activating the SAPK/JNK pathway, which increases the transcription of proapoptotic genes under the control of the transcription factor c-Jun. Caspases can also modulate the activity of specific proteins by inactivating inhibitors of these proteins, such as DNAse CAD/DFF40, which is constantly inactivated by its inhibitor ICAD. This inhibitor is a caspase 3 substrate, and CAD/DFF40 release results in chromosome cleavage at internucleosomal spaces. These irreversible proteolytic events are responsible for the morphological changes displayed by apoptotic cells.

Caspase activation occurs as a late and common step in all cells undergoing apoptosis. Nevertheless, there are many initial pathways that can result in caspase activation. Probably, each distinct pathway is triggered by different apoptotic stimuli. In mammalian cells, the apoptotic response is usually mediated by the intrinsic and extrinsic pathways, depending on the origin of the death signal. The intrinsic pathway can further be divided into mitochondrial and ER stress pathways.

The Bcl-2 family and the intrinsic mitochondrial pathway

Besides its role as the energy-generating organelle, the mitochondrion has recently emerged as the center of conversion of cellular life and death signals. This organelle contains, in its intermembrane space, apoptogenic

factors, like cytochrome *c*, AIF (apoptosis-inducing factor), procaspases 2, 3, and 9, Smac/DIABLO (second mitochondrial activator of caspases/direct inhibition of apoptosis protein IAP binding protein with low pI), Omi/HtrA2, and endonuclease G (Gross *et al*., 1999). In the presence of apoptotic signals, these factors are released into the cytoplasm and a few of them participate in caspase activation. This apoptotic pathway centered on the mitochondria is known as the intrinsic mitochondrial or mitochondria-dependent caspase activation pathway.

Proteins of the Bcl-2 family are responsible for the maintenance or release of these factors from the mitochondria into the cytoplasm. For this reason, this family and the caspase family are considered as the main regulators of the apoptosis process (Gross *et al*., 1999; Cory and Adams, 2002; Kuwana and Newmeyer, 2003). To date, at least 20 members of the Bcl-2 family have been identified, which can be divided into two main groups, depending on their function. The proapoptotic group contains members (e.g. Bax, Bcl-X_S, Bak, Bad, Bid, Bik) that induce the release of apoptogenic factors from the mitochondria into the cytoplasm, resulting in apoptosis. On the other hand, the antiapoptotic group contains proteins (e.g. Bcl-2, Bcl-X_L, Mcl-1, Bcl-w, Boo) that are responsible for the maintenance of these factors inside the mitochondria, inhibiting the apoptotic process (Adams and Cory, 1998).

Members of the Bcl-2 family share one or more Bcl-2 homology (BH) domains, named BH1, BH2, BH3, and BH4 (Adams and Cory, 1998). It is not yet clear which structural features determine if these proteins possess pro- or anti-apoptotic activities. However, some studies revealed that the BH3 domain is a critical domain for the proapoptotic members (Chittenden *et al*., 1995). Besides BH domains, some contain a hydrophobic domain in the C-terminal region, which is essential for the attachment to intracellular membranes, like the outer mitochondrial, nuclear, and endoplasmic reticulum membranes (Krajewski *et al*., 1993; Nguyen *et al*., 1993).

In the absence of a death signal, most of the pro- and anti-apoptotic members are located in separate subcellular compartments. Anti-apoptotic proteins are inserted in intracellular membranes, mainly the mitochondrial membrane, while some proapoptotic members are located in the cytoplasm or cytoskeleton in an inactive form. They are activated and translocated by apoptotic stimuli to their place of action to perform their functions (Gross *et al*., 1999).

A remarkable feature of the Bcl-2 proteins is their ability to interact with one another to form either homo- or hetero-dimers (Oltvai *et al*., 1993). The formation of hetero-dimers between pro- and anti-apoptotic members suggests a competitive neutralization of their activities. A healthy cell maintains a balance between these groups of proteins and a destabilization in their relative concentration determines, at least in part, the decision for cell survival or cell death (Gross *et al*., 1999).

Proapoptotic proteins can be further classified according to their BH domains (*Figure 7.5*). Some members, such as Bax and Bak, contain multiple domains and others, like Bid, Bad, Bim, and Bmf, contain only the BH3 domain (Gross *et al*., 1999; Kuwana and Newmeyer, 2003). These structural differences also reflect differences in their function. Multidomain proteins directly induce outer mitochondrial membrane permeabi-

lization with consequent release of apoptogenic factors into the cytoplasm (Desagher et al., 1999; Zong *et al*., 2001). On the other hand, the proteins with only the BH3 domain are considered the essential initiators of the cell death program (Huang and Strasser, 2000; Bouillet and Strasser, 2002). When activated by apoptotic stimuli, the BH3-only proteins have two fates: (1) some are targeted to the outer mitochondrial membrane and heterodimerize with Bcl-2 and Bcl-X_L, neutralizing the action of these

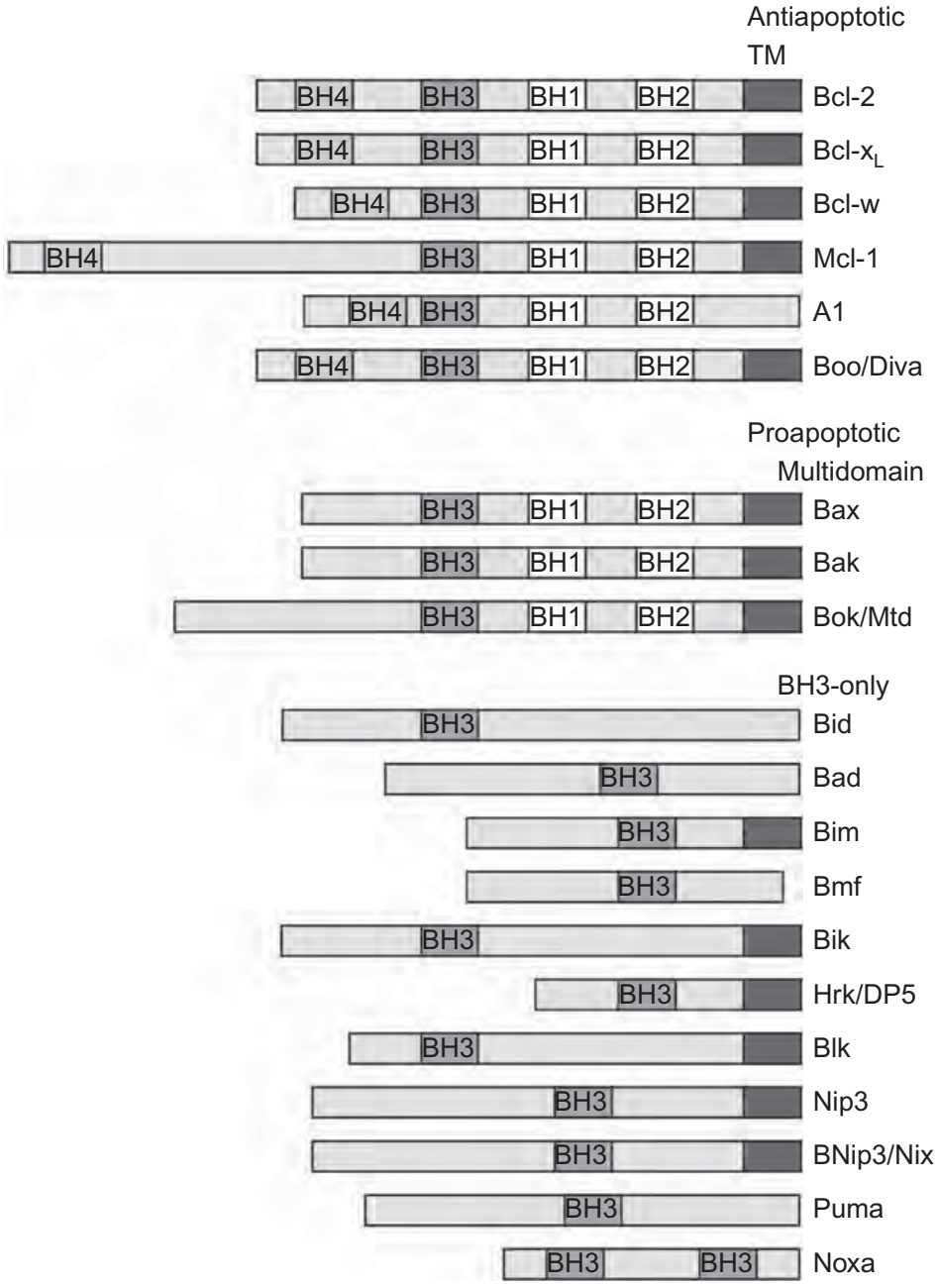


Figure 7.5

Classification scheme of the Bcl 2 family members. TM refers to the hydrophobic C terminal region, which is probably a transmembrane domain (adapted from Kuwana and Newmeyer, 2003).

antiapoptotic proteins and allowing, indirectly, multidomain proapoptotic Bax and Bak proteins to release apoptogenic factors from the mitochondria; (2) others, besides the previously described function, are also responsible for the direct activation of the multidomain proteins, as is the case for Bid (Desagher *et al*., 1999; Eskes *et al*., 2000) and probably Bim (Letai *et al*., 2002). At least two events seem to be critical for Bax and Bak to release apoptogenic factors into the cytoplasm homodimerization and insertion into the outer mitochondrial membrane (Bouillet and Strasser, 2002).

It is not clear whether each member of this subgroup is activated by a particular stimulus and through a specific mechanism, or whether their roles are redundant. Nevertheless, it is possible that different BH3-only domain proteins, or their combinations, are critical for apoptosis in different cell types.

Tridimensional structure analysis of some Bcl-2 family members, such as Bcl-X_L, Bcl-w, Bax, and Bid, surprisingly revealed that pro- and antiapoptotic proteins share common structures (Kuwana and Newmeyer, 2003). The BH3 domain is buried inside the molecule, and it has been suggested that it is essential for activity of the proapoptotic members and has to be exposed to render the protein active. Therefore, in a healthy cell, proapoptotic members are inactive, with the BH3 domain hidden inside the molecule. However, by receiving apoptotic signals, they undergo a conformational change, exposing this domain and, thus, acquiring proapoptotic activity.

In some molecules, such as Bid, Bax, Bak, Bmf, and Bim, the N-terminal region acts as an inhibitory domain, hiding the BH3 domain (Gross *et al*., 1999). Bid must be cleaved by caspase 8, and its truncated form (without the N-terminal) translocates to the mitochondria to interact with Bax and/or Bak, activating them (Li *et al*., 1998; Desagher *et al*., 1999). Prior to the apoptotic signal, Bmf and Bim are found associated with cytoskeleton complexes by the N-terminal region. In the presence of these signals, they dissociate from these complexes and translocate to the mitochondria to bind Bcl-2 and Bcl-X_L, antagonizing their antiapoptotic activity (Puthalakath *et al*., 1999, 2001). Bax and Bak require the interaction with some BH3-only proteins to derepress their N-terminal domain, exposing not only their BH3-only domain, but also a C-terminal hydrophobic domain, which allows them to become integral proteins in the outer mitochondrial membrane and induce the release of apoptogenic factors (Goping *et al*., 1998; Desagher *et al*., 1999). Bad is phosphorylated at two serine residues (Ser-112 and Ser-136), which allows it to be sequestered by the cytosolic protein 14-3-3, keeping it inactivated (Zha *et al*., 1996). In the presence of apoptotic signals, Bad is dephosphorylated, resulting in its dissociation from the 14-3-3 protein and its translocation to the outer mitochondrial membrane to bind to Bcl-2 and Bcl-X_L (Kelekar *et al*., 1997; Ottilie *et al*., 1997). It is suggested that Bad phosphorylation regulates the BH3 domain exposure (Zha *et al*., 1997). Antiapoptotic proteins can also be converted to proapoptotic if they expose their BH3 domains (Cheng *et al*., 1997).

However, not all proapoptotic members are regulated post-translationally. Some, such as Noxa, Puma, and HRK, are regulated transcriptionally. Noxa and Puma are regulated by the p53 protein and, therefore, are critical

for an apoptosis process induced by DNA damage (Oda *et al*., 2000; Nakano and Vousden, 2001), while HRK is regulated by the JNK-dependent mechanism (Harris and Johnson, 2001). Some post-translationally regulated proteins can also be regulated transcriptionally, such as Bax, which can also be regulated by p53 (Miyashita and Reed, 1995). Antiapoptotic members, like Bcl-X_L, Mcl-1, A-1 and, less frequently, Bcl-2, can also be regulated transcriptionally (Gross *et al*., 1999).

The exact manner of how proapoptotic proteins induce the release of apoptogenic factors from the mitochondrial intermembrane space into the cytoplasm and how antiapoptotic proteins prevent it remains obscure.

The protection conferred by antiapoptotic members may occur by their direct binding to proapoptotic members, sequestering the BH3-only proteins and therefore preventing the activation of Bax and Bak or directly neutralizing the activity of the multidomain proteins (Oltvai and Korsmeyer, 1994). The heterodimerization between members of Bcl-2 family occurs by the insertion of the BH3 domain of the proapoptotic protein into a hydrophobic pocket formed by the BH1, BH2, and BH3 domains on the surface of the antiapoptotic protein (Sattler *et al*., 1997). Bcl-2 and Bcl-X_L do not prevent Bid-induced conformational change of Bax and Bak (Desagher *et al*., 1999). However, they block the release of apoptogenic factors from the mitochondria. Some mutants of Bcl-X_L that have lost the ability to form heterodimers with Bax and Bak can still suppress cell death by apoptosis, suggesting the existence of a protection mechanism independent of heterodimer formation (Cheng *et al*., 1996).

In the literature, two main mechanisms have been proposed that could explain how Bax and Bak induce the release of apoptogenic factors, especially cytochrome *c*. The first model of outer mitochondrial membrane permeabilization predicts the occurrence of homo-oligomerization of Bax (probably four molecules) and Bak, resulting in the formation of channels just wide enough for the passage of cytochrome *c*. The passage of other apoptogenic factors is still contested (Antonsson *et al*., 1997; Saito *et al*., 2000; Kuwana *et al*., 2002; Kuwana and Newmeyer, 2003). The second model is based on the activity regulation of pre-existent channels, such as the permeability transition pore (PTP) (Marzo *et al*., 1998; Narita *et al*., 1998; Kuwana and Newmeyer, 2003). PTP is a multiprotein channel, formed by components of both the outer and inner mitochondrial membranes and matrix proteins, including VDAC (voltage-dependent anion channel, also known as mitochondrial porin), ANT (adenine nucleotide translocator), and cyclophilin D, respectively (Crompton *et al*., 1999). Bax, Bak, and Bcl-X_L have been found to interact with VDAC (Narita *et al*., 1998; Shimizu *et al*., 1999), and Bax to interact with ANT (Marzo *et al*., 1998). The PTP opening would be followed by the swelling of the mitochondrial matrix and the rupture of the outer membrane. In this context, the release of apoptogenic factors would not be specific and would occur indirectly, as a result of the rupture of the outer membrane. When PTP is induced, the inner mitochondrial membrane potential ($\Delta\psi$m) becomes dissipated, leading to the loss of mitochondrial functions, such as energy production and protein import.

Some authors suggest that the mitochondria, especially the inner membrane, remain intact during apoptosis. Thus, it is suggested that PTP activation is not involved or that cells starting to die by apoptosis could switch to necrosis, therefore activating the PTP (Antonsson, 2001). Nevertheless, it is important to remember that these contradictory results can occur since apoptosis can be activated by distinct mechanisms in different cell types by different apoptotic signals. It is also possible that both mechanisms are correct. Bax and Bak oligomers can form channels for the initial release of cytochrome *c*, followed by a larger flux through PTP. In both cases, antiapoptotic proteins Bcl-2 and Bcl-X_L seem to inhibit the formation of both kinds of channels.

Following the outer mitochondrial membrane permeabilization, the apoptogenic factors are released into the cytoplasm. Among them, cytochrome *c* has an important role in caspase activation, because it is the cofactor for assembling a large caspase 9 activating complex in the cytoplasm, called apoptosome. Along with cytochrome *c*, the Apaf-1 protein and dATP or ATP are required to form this complex in the cytoplasm (Hill *et al*., 2003).

Apaf-1 consists of three functional domains: an N-terminal CARD, a central NBD (nucleotide-binding domain), and WD-40 repeats at the C-terminal region (*Figure 7.6*). In the absence of cytochrome *c*, Apaf-1 exists as a monomer in a compact, auto-inhibited form (Hu *et al*., 1998; Acehan *et al*., 2002). When cytochrome *c* and dATP (or ATP) are present, Apaf-1 is forced into a more open conformation, facilitating the oligomerization with adjacent Apaf-1 molecules (Jiang and Wang, 2000) and the cytochrome *c* association with the WD-40 repeats (Acehan *et al*., 2002). The apoptosome assembly is illustrated in *Figure 7.6*.

It has been suggested that the apoptosome is formed by the oligomerization of seven Apaf-1 molecules, resulting in a wheel-like structure. This structure comprises a central hub connected to seven radial spokes, as shown in *Figure 7.6*. The model suggests that the ring is composed of seven Apaf-1 CARD domains held together in close proximity. Apaf-1 central and C-terminal regions form spokes projecting outward from the hub (Qin *et al*., 1999; Acehan *et al*., 2002). The procaspase 9 is recruited into the apoptosome through interaction with Apaf-1 with the procaspase 9 CARD domains, at a 1:1 proportion (Budihardjo *et al*., 1999; Jiang and Wang, 2004). The procaspase 9 aggregation leads to autoproteolysis (Saleh *et al*., 1999). Caspase 9 and the apoptosome form an active holoenzyme, responsible for the activation of downstream effector caspases, such as caspases 3 and 7 (Bratton *et al*., 2001).

After caspase 9 activation, the death signal is propagated by downstream caspase activation. Caspase 9 directly activates caspases 3 and 7. Caspase 3, in turn, processes and activates caspases 2 and 6 and also caspase 9, therefore amplifying the death signal. Caspase 6 cleaves and activates caspases 8 and 10 (Slee *et al*., 1999).

Besides cytochrome *c* and procaspase 9, other important apoptogenic factors are also released from the mitochondria, such as SMAC/DIABLO, Omi/HrtA2, AIF, and endonuclease G. The function of SMAC/DIABLO and Omi/HrtA2 is to activate caspase by suppressing the caspase inhibitory activity of IAP (Du *et al*., 2000; Verhagen *et al*., 2000). The protein

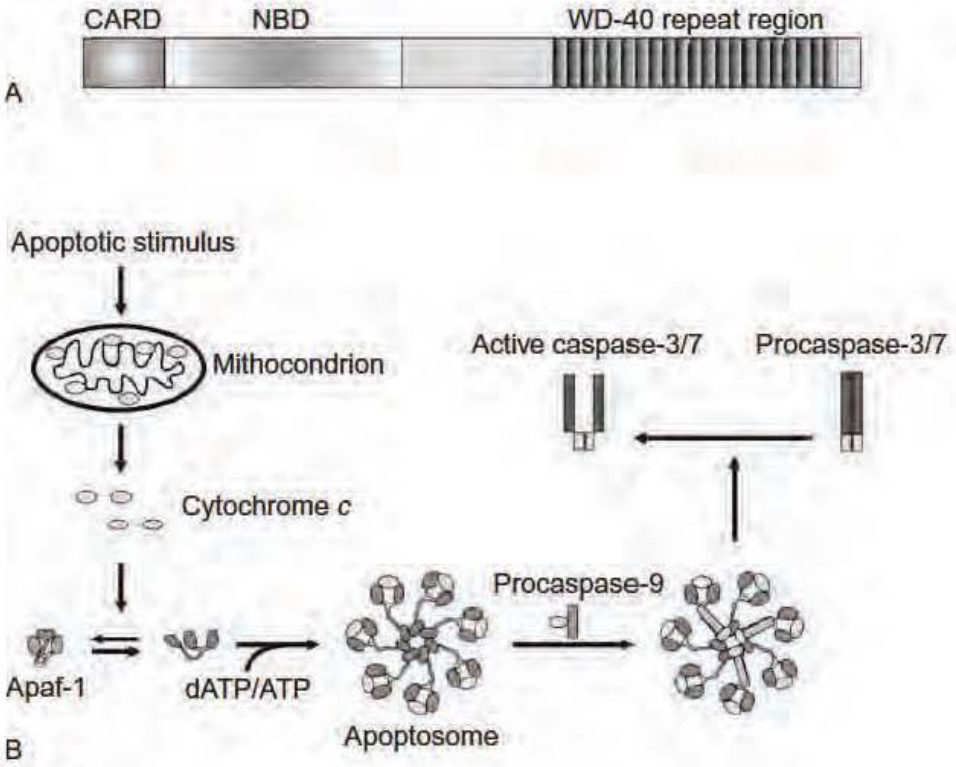


Figure 7.6

Scheme of the mechanism of apoptosome formation. (A) The Apaf 1 molecule possesses three domains: CARD, NBC, and WD 40 repeats. (B) In the absence of cytochrome *c*, Apaf 1 may exist in a compact, autoinhibited form, with the CARD region buried between the lobes of WD 40 repeats. The binding of cytochrome *c* displaces the CARD region from the WD 40 repeats, forcing the molecule into a more open conformation. The interaction with dATP/ATP prevents the reassociation of CARD to the lobes of WD 40 repeats, facilitating the interaction with other Apaf 1 molecules for the apoptosome assembly. Procaspases 9 are recruited by a CARD:CARD interaction, leading to their autoprocessing (adapted from Hill *et al.*, 2003, and Jiang and Wang, 2004).

family IAP, which contains members like survivin, xIAP, cIAP1, cIAP2, inhibits caspase activity by directly binding to the active enzymes. These proteins contain one or more BIR (baculovirus IAP repeat) domains, which are responsible for the caspase inhibitory activity. In healthy cells, it is likely that IAP proteins serve to inhibit residual or unwanted caspase activity. SMAC/DIABLO directly binds to BIR domains of IAPs, inhibiting their functions. Since Omi/HtrA2 is a serine-protease, it proteolytically cleaves and inactivates IAP proteins.

When AIF and endonuclease G are released into the cytoplasm, they directly translocate to the nucleus and induce DNA fragmentation and subsequent chromosomal condensation, a remarkable morphological feature of the apoptotic process. AIF induces chromatin digestion into large fragments of approximately 50 kb, probably by activating a nuclear DNAse. Therefore, these proteins are important for the caspase-independent apoptosis pathway.

The intrinsic ER stress-induced pathway

The ER is an organelle in which most proteins acquire their tertiary and quaternary structures. It is also the Ca^{2+} storage site (Rao *et al*., 2004). Under certain conditions, such as disruption of Ca^{2+} homeostasis, hypoxia or ischemia, or when there is an overload of proteins, an accumulation of unfolded proteins occurs in the ER. This accumulation activates a compensatory mechanism called ER stress response or unfolded protein response (UPR). This response consists of four distinct steps: a cell cycle arrest in G_1/S phase; attenuation of protein synthesis to prevent further protein aggregation and accumulation in this organelle; induction of ER-localized chaperone proteins to assist protein folding; and activation of a protein degradation mechanism to eliminate unwanted protein aggregates. However, if the ER stress cannot be bypassed, it culminates in apoptosis (Szegezdi *et al*., 2003).

Since this ER pathway was discovered recently, not much is known about its signaling mechanism. However, it is clear that this pathway can unleash the apoptotic process through three distinct mechanisms. The first mechanism is dependent on a transcription factor, the GADD153/CHOP. Although no target genes have been identified to date, it has been speculated that GADD153/CHOP can decrease Bcl-2 expression (McCullough *et al*., 2001).

The second mechanism is dependent on caspase 12 activation. Nevertheless, it is still not clear how this activation occurs. Preliminary studies showed a caspase activating complex containing VCP and ALG-2 proteins, which also possess apoptotic activities. This has suggested the existence of a caspase 12 activating complex, similar to the apoptosome, which was designated eraptosome (Hoppe *et al*., 2002; Kilic *et al*., 2002). The adaptor protein TRAF2 may be involved in procaspase 12 aggregation, resulting in its cleavage and activation (Yoneda *et al*., 2001). The procaspase 12 may also be processed by caspase 7, through a cleavage in the middle of its prodomain, which leads to autoprocessing between the prodomain and the large subunit, and between the large and small subunits of caspase 12 (Szegezdi *et al*., 2003). Furthermore, it was also suggested that caspase 12 can be processed by calpain. The disruption of Ca^{2+} homeostasis leads to calpain activation, which in turn translocates from the cytoplasm to the ER and cleaves off the caspase 12 CARD prodomain. After this processing, this caspase is autoprocessed into large and small subunits (Szegezdi *et al*., 2003). Once activated, caspase 12 cleaves and activates the procaspase 9, triggering the remaining caspase proteolytic cascade. Calpain can also cleave Bid.

The third mechanism is Ca^{2+}-dependent and also involves the intrinsic mitochondrial pathway. Some apoptotic stimuli induce Ca^{2+} release from the ER, and this process may be regulated by the Bcl-2 family members that reside in this organelle (Szegezdi *et al*., 2003). Bax and Bak seem to induce Ca^{2+} release, while antiapoptotic members, such as Bcl-2, seem to reduce this process. Ca^{2+} ions released from the ER eventually accumulate in mitochondria, which induces permeabilization of the outer mitochondrial membrane through PTP formation. Release of apoptogenic factors

into the cytoplasm causes activation of caspase 9 and mitochondria-mediated apoptosis. Apoptogenic Bap31 protein, which resides in the ER, can be cleaved by caspase 8 and also seems to induce Ca^{2+} release from this organelle (Breckenridge *et al*., 2003).

The extrinsic or death receptor-induced pathway

The extrinsic pathway consists of a series of events initially induced by death receptors located on the cell surface. It is initiated by interaction of extracellular death ligands with their respective receptors, located on the surface of the plasma membrane. The death ligands are members of the tumor necrosis factor (TNF)/nerve growth factor (NGF) superfamily. TNF-R1, Fas (Apo-1/CD95), TRAIL-R1, TRAIL-R2, and NGF-R are examples of death receptors. They are transmembrane proteins consisting of an external domain, where the ligand associates, and a cytoplasmic domain, which contains the DD (death domain).

The death ligands, such as FasL, are typically homotrimeric molecules. When they bind to their receptors, which are monomers, they induce aggregation and trimerization of the receptors, resulting in their cytoplasmic domains becoming physically closer together. The close proximity of the DDs results in the recruitment of adaptor proteins located in the cytoplasm, such as FADD and TRADD. These adaptor proteins also possess a DD and bind to the DD of the death receptors. FADD binds mainly to Fas, while TRADD binds preferentially to TNF-R1. After TRADD binds to TNF-R1, FADD can bind to TRADD. FADD also possesses another domain, besides DD, which is called DED. Procaspases 8 and 10 (initiator caspases) also possess the DED and are able to bind to the adaptor protein through their DED, therefore assembling the DISC (death-inducing signaling complex) (*Figure 7.7*).

The DISC-induced procaspase assembly results in the autoactivation of caspases 8 and 10. The DISC process of caspase activation seems to be analogous to the apoptosome process of caspase 9 activation. Caspase 8, in turn, cleaves and activates caspase 3, which is responsible for the apoptotic signal amplification with subsequent cell collapse.

Some cell types maintain a low level of caspase 8 in the cytoplasm. Therefore, in the presence of apoptotic stimuli, this small amount of caspase 8 is activated in the DISC complex, and the subsequent activation of effector caspases is not possible. These cell types are called type II and also require the mitochondrial pathway activation. These two pathways can communicate through the cleavage of a Bcl-2 member, Bid, by caspase 8 (Li *et al*., 1998). Cell lines that have a higher level of caspase 8 in the cytoplasm are called type I. In these cells, Bcl-2 family members do not regulate the death receptor-mediated pathway.

The proteolytic cleavage of Bid removes its N-terminal portion, exposing the BH3 domain (Li *et al*., 1998). The truncated Bid protein, called tBid or p15, translocates to the mitochondria, where it interacts with Bax or Bak, inducing a conformational change in these proteins (Desagher *et al*., 1999). This conformational change is necessary for the permeabilization of the outer mitochondrial membrane and the subsequent release of the apoptogenic factors into the cytoplasm, resulting in caspase 9 activation.

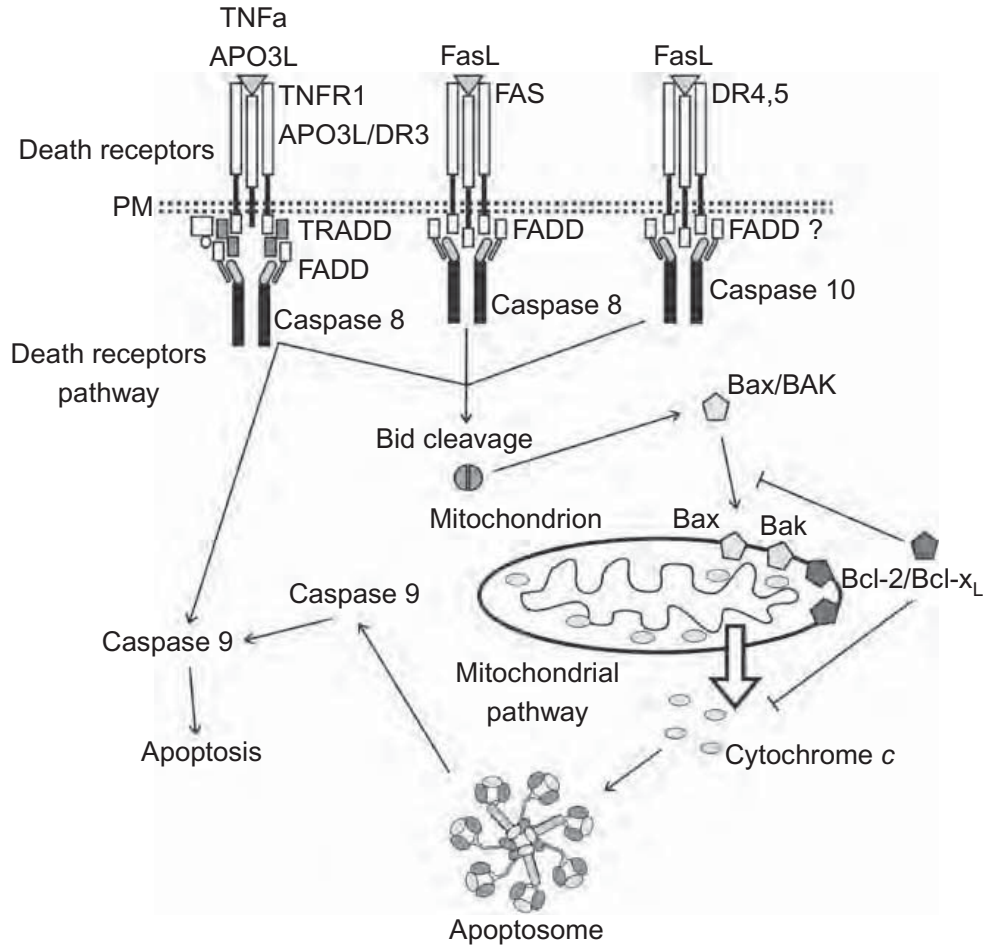


Figure 7.7

Caspase activation through the death receptor induced pathway. The activation of initiator caspases 8 and 10 by the death receptors results in the activation of effector caspases 3, 6, and 7. In type II cell lines, the activation of these initiator caspases also results in Bid cleavage and, therefore, in the activation of the mitochondrial pathway.

7.6.2 Molecular strategies for apoptosis control

Since apoptosis occurs under the control of several genes, the molecular manipulation of the signaling cascade could block apoptosis progression and prolong cell viability. Maintaining a high cell viability in bioreactors is of great interest for biotechnological processes. Therefore, many researchers in this area are devoted to the development of genetically modified cell lines with increased resistance to apoptosis.

The most common strategies consist of developing recombinant cell lines expressing antiapoptotic genes that regulate the two main families of proteins involved in the apoptotic cascade: the Bcl-2 and caspase families.

The Bcl-2 gene has been the most widely studied and its overexpression in cells has been described. It has the ability to protect several industrially important cell lines, such as hybridomas, myelomas and CHO, BHK, and COS cells, against apoptotic stimuli that are typical of a cell culture

process, such as nutrient deprivation, serum withdrawal, oxygen limitation, hydrodynamic stress, accumulation of toxic metabolites, and viral infection. In almost all cases, this overexpression prolonged cell viability. However, eventually, the cultures displayed different phenotypes. In some cases, the cell density increased, but in others a growth arrest was observed (Vives *et al*., 2003a). Moreover, the effect of this overexpression in the productivity of recombinant proteins is variable. In some cases, an increase in productivity was observed, while in other cases there was no significant effect or even a decrease in productivity (Vives *et al*., 2003a; Tey and Al-Rubeai, 2004). In most cases, the level of Bcl-2 expression correlates with the level of protection. Nevertheless, the expression of Bcl-2 has shown little to no apoptosis protection in some cell types, as in the case of HeLa, NS0, and insect (Sf9) cells (Vives *et al*., 2003a). Overexpression of Bcl-X_L has also been efficient in the control of apoptosis (Charbonneau and Gauthier, 2000; Mastrangelo *et al*., 2000a, 2000b).

The cells probably have an intrinsic regulation mechanism for the level of antiapoptotic proteins. In the Bcl-2 case, the regulation occurs through the action of caspase 3, resulting in a 23 kDa fragment with proapoptotic activity. Therefore, the overexpression of Bcl-2, and probably Bcl-X_L, may not be completely efficacious, because at some point these proteins will be degraded or even modified into proapoptotic proteins. To bypass this situation, a Bcl-2 mutant, resistant to the intrinsic cell regulation, was expressed in CHO and BHK cells and showed improved efficacy over the wild-type protein (Figueroa *et al*., 2001). Also, some viral Bcl-2 homolog genes, such as E1B-19kDa (from adenovirus), BHRF1 (from Epstein–Barr virus), and KSBcl-2 (from Kaposi's sarcoma-associated herpesvirus) have been studied, since they are resistant to the intrinsic cell regulation (Vives *et al*., 2003b). Although the overexpression of Bcl-2 gene could not protect NS0 cells from apoptosis, the expression of the E1B-19kDa analog could. This suggests a different mechanism of action between E1B-19kDa and Bcl-2 in inhibiting apoptosis (Mercille *et al*., 1999).

There are just a few studies of the use of caspase inhibitors to prevent apoptosis. Most studies concentrate on the expression of proteins of the IAP family (XIAP being the most noticeable) and the viral components p35 and CrmA (Vives *et al*., 2003a). CrmA, encoded by the smallpox virus, is a pseudo-substrate for serine and cysteine proteases. It inhibits caspases 1, 8, and 10 in several cell types (Sauerwald *et al*., 2003). p35 is a wide-spectrum caspase inhibitor encoded by baculoviruses, and it also behaves as a pseudo-substrate, inhibiting caspases 1, 3, 6, 7, 8, and 10 (Zhou *et al*., 1998). XIAP is the most potent member of the IAP family. It is found in the mammalian genome and is responsible for the inhibition of caspases 3, 7, and 9 (Sauerwald *et al*., 2002). An increased protective effect is found in CHO and HEK-293 cells expressing a XIAP mutant resistant to degradation (XIAP-BIR123) when compared with the wild-type protein (Sauerwald *et al*., 2002).

Numerous pathways are involved in the activation of apoptosis, although at some point, all these pathways seem to converge to a few related ones. In order to be more efficient, molecular strategies to prevent apoptosis should concentrate on these few steps of the apoptotic cascade that are common to all cell types and to different apoptotic stimuli. For

this reason, research on genetic manipulation to prevent apoptosis is focusing on the last steps of the cascade. While many strategies have enabled a delay of the apoptotic process, so far none is capable of completely circumventing it. This suggests that the cells may have other mechanisms available to overcome the antiapoptotic activity of the expressed proteins. It is also possible that the heterologous gene is lost during cellular division or that death by other mechanisms, such as necrosis, is responsible for the inability to completely circumvent cell death.

7.7 Conclusions and perspectives

Complex phenomena regulate cell proliferation and cell death both *in vivo* and *in vitro*. The increasing understanding of these phenomena is serving as a basis for studies aimed at improving the performance of biotechnological processes based on *in vitro* animal cell culture. Multidisciplinary research projects, involving groups from different disciplines, such as biology and engineering, are advancing towards the development of techniques to control cell proliferation and cell death, with the aim of increasing the longevity and robustness of cells in culture. The outcome of these studies will constitute, in the future, important tools to increase productivity, predictability, and reliability of animal cell culture processes.

References

Acehan D, Jiang X, Morgan DG, Heuser JE, Wang X, Akey CW (2002), Three dimensional structure of the apoptosome: implications for assembly, procaspase 9 binding, and activation, Mol. Cell. 9:423 432.

Adams JM, Cory S (1998), The Bcl 2 protein family: arbiters of cell survival, Science 281:1322 1326.

Alberts B, Johnson A, Lewis J, Raff M, Roberts K, Walter P (2002), Molecular Biology of the Cell, 4th Ed., Garland Science, New York.

Alnemri ES, Livingston DJ, Nicholson DW, Salvesen G, Thornberry NA, Wong WW, Yuan J (1996), Human ICE/CED 3 protease nomenclature, Cell 87:171.

Al Rubeai M (1998), Apoptosis and cell culture technology, Adv. Biochem. Eng. Biotechnol. 59:225 249.

Al Rubeai M, Singh RP (1998), Apoptosis in cell culture, Curr. Opin. Biotechnol. 9:152 156.

Al Rubeai M, Emery AN, Chalder S, Jan DC (1992), Specific antibody productivity and the cell cycle comparisons of batch, continuous and perfusion cultures, Cytotechnology 45:463 472.

Al Rubeai M, Singh RP, Goldman MH, Emery AN (1995a), Death mechanisms of animal cells in conditions of intensive agitation, Biotechnol. Bioeng. 45:463 472.

Al Rubeai M, Singh RP, Emery AN, Zang Z (1995b), Cell cycle and cell size dependence of susceptibility to hydrodynamic forces, Biotechnol. Bioeng. 46: 88 92.

Antonsson B (2001), Bax and other pro apoptotic Bcl 2 family ''killer proteins'' and their victim, the mitochondrion, Cell Tissue Res. 306:347 361.

Antonsson B, Conti F, Ciavatta A, Montessuit S, Lewis S, Martinou I, Bernasconi L, Bernard A, Mermod JJ, Mazzei G, Maundrell K, Gambale F, Sadoul R, Martinou JC (1997), Inhibition of Bax channel forming activity by Bcl 2, Science 277: 370 372.

Arden N, Betenbaugh MJ (2004), Life and death in mammalian cell culture: strategies for apoptosis inhibition, Trends Biotechnol. 22:174 180.

Bekaert S, Derradji H, Baatout S (2004), Telomere biology in mammalian germ cells and during development, Dev. Biol. 274:15 30.

Bouillet P, Strasser A (2002), BH3 only proteins evolutionarily conserved proapop totic Bcl 2 family members essential for initiating programmed cell death, J. Cell Sci. 115:1567 1574.

Bratton SB, Walker G, Srinivasula SM, Sun XM, Butterworth M, Alnemri ES, Cohen GM (2001), Recruitment, activation and retention of caspases 9 and 3 by Apaf 1 apoptosome and associated XIAP complexes, EMBO J. 20:998 1009.

Breckenridge DG, Stojanovic M, Marcellus RC, Shore GC (2003), Caspase cleavage product of BAP31 induces mitochondrial fission through endoplasmic reticulum calcium signals, enhancing cytochrome c release to the cytosol, J. Cell Biol. 160:1115 1127.

Brown DG, Sun X M, Cohen GM (1993), Dexamethasone induced apoptosis involves cleavage of DNA to large fragments prior to internucleosomal fragmentation, J. Biol. Chem. 268:3037 3039.

Budihardjo I, Oliver H, Lutter M, Luo X, Wang X (1999), Biochemical pathways of caspase activation during apoptosis, Annu. Rev. Cell Dev. Biol. 15:269 290.

Buja LM, Eigenbrodt ML, Eigenbrodt EH (1993), Apoptosis and necrosis. Basic types and mechanisms of cell death, Arch. Pathol. Lab. Med. 117:1208 1214.

Calle Y, Palomares T, Castro B, del Olmo M, Alonso Varona A (2001), Removal of N glycans from cell surface proteins induces apoptosis by reducing intracellular glutathione levels in the rhabdomyosarcoma cell line S4MH, Biol. Cell. 92: 639 646.

Charbonneau JR, Gauthier ER (2000), Prolongation of murine hybridoma cell survival in stationary batch culture by Bcl XL expression, Cytotechnology 34:131 139.

Cheng EH, Levine B, Boise LH, Thompson CB, Hardwick JM (1996), Bax indepen dent inhibition of apoptosis by Bcl XL, Nature 379:554 556.

Cheng EH, Kirsch DG, Clem RJ, Ravi R, Kastan MB, Bedi A, Ueno K, Hardwick JM (1997), Conversion of Bcl 2 to a Bax like death effector by caspases, Science 278:1966 1968.

Chittenden T, Flemington C, Houghton AB, Ebb RG, Gallo GJ, Elangovan B, Chinnadurai G, Lutz RJ (1995), A conserved domain in Bak, distinct from BH1 and BH2, mediates cell death and protein binding functions, EMBO J. 14: 5589 5596.

Cory S, Adams JM (2002), The Bcl2 family: regulators of the cellular life or death switch, Nat. Rev. Cancer 2:647 656.

Cotter TG, Al Rubeai M (1995), Cell death (apoptosis) in cell culture systems, Trends Biotechnol. 13:150 155.

Crompton M, Virji S, Doyle V, Johnson N, Ward JM (1999), The mitochondrial permeability transition pore, Biochem. Soc. Symp. 66:167 179.

Darmon AJ, Nicholson DW, Bleackley RC (1995), Activation of the apoptotic protease CPP32 by cytotoxic T cell derived granzyme B, Nature 377:446 448.

Desagher S, Osen Sand A, Nichols A, Eskes R, Montessuit S, Lauper S, Maundrell K, Antonsson B, Martinou JC (1999), Bid induced conformational change of Bax is responsible for mitochondrial cytochrome c release during apoptosis, J. Cell Biol. 144:891 901.

Dickson AJ (1998), Apoptosis regulation and its application to biotechnology, Trends Biotechnol. 16:339 342.

Du C, Fang M, Li Y, Li L, Wang X (2000), Smac, a mitochondrial protein that promotes cytochrome c dependent caspase activation by eliminating IAP inhibi tion, Cell 102:33 42.

Earnshaw WC, Martins LM, Kaufmann SH (1999), Mammalian caspases: structure,

activation, substrates, and functions during apoptosis, Annu. Rev. Biochem. 68:383 424.
Eskes R, Desagher S, Antonsson B, Martinou JC (2000), Bid induces the oligomeriza tion and insertion of Bax into the outer mitochondrial membrane, Mol. Cell Biol. 20:929 935.
Figueroa B Jr, Sauerwald TM, Mastrangelo AJ, Hardwick JM, Betenbaugh MJ (2001), Comparison of Bcl 2 to a Bcl 2 deletion mutant for mammalian cells exposed to culture insults, Biotechnol. Bioeng. 73:211 222.
Fussenegger M, Bailey JE (1998), Molecular regulation of cell cycle and apoptosis in mammalian cells: implications for biotechnology, Biotechnol. Prog. 14:807 833.
Fussenegger M, Bailey JE (1999), Control of mammalian cell proliferation as an important strategy in cell culture technology, In: Al Rubeai M, Dordrecht AH (Eds), Cancer Therapy and Tissue Engineering, Kluwer, Dordrecht.
Fussenegger M, Bailey JE, Hauser H, Mueller PP (1999), Genetic optimization of recombinant glycoprotein production by mammalian cells, Trends Biotechnol. 17:35 42.
Goping IS, Gross A, Lavoie JN, Nguyen M, Jemmerson R, Roth K, Korsmeyer SJ, Shore GC (1998), Regulated targeting of BAX to mitochondria, J. Cell Biol. 143:207 215.
Griffiths JB (1984), Cell biology: experimental aspects, In: Spier RE, Griffiths JB (Eds), Animal Cell Biotechnology, Chapter 3, Academic Press, Orlando.
Gross A, McDonnell JM, Korsmeyer SJ (1999), BCL 2 family members and the mitochondria in apoptosis, Genes Dev. 13:1899 1911.
Harris CA, Johnson EM Jr (2001), BH3 only Bcl 2 family members are coordinately regulated by the JNK pathway and require Bax to induce apoptosis in neurons, J. Biol. Chem. 276:37754 37760.
Harris SL, Levine AJ (2005), The p52 pathway: positive and negative feedback, Oncogene 24:2899 2908.
Hayflick L (1965), The limited *in vitro* lifetime of human diploid cell strains, Exp. Cell Res. 37:614 636.
Hayflick L, Moorhead PS (1961), The serial cultivation of human diploid cell strains, Exp. Cell Res. 25: 585 621.
Hill MM, Adrain C, Martin SJ (2003), Portrait of a killer: the mitochondrial apopto some emerges from the shadows, Mol. Interv. 3:19 26.
Hofmann K, Bucher P, Tschopp J (1997), The CARD domain: a new apoptotic signaling motif, Trends Biochem. Sci. 22:155 156.
Hoppe J, Kilic M, Hoppe V, Sachinidis A, Kagerhuber U (2002), Formation of caspase 3 complexes and fragmentation of caspase 12 during anisomycin induced apoptosis in AKR 2B cells without aggregation of Apaf 1, Eur. J. Cell Biol. 81:567 576.
Horvitz HR, Shaham S, Hengartner MO (1994), The genetics of programmed cell death in the nematode *Caenorhabditis elegans*, Cold Spring Harb. Symp. Quant. Biol. 59:377 385.
Hu Y, Ding L, Spencer DM, Nunez G (1998), WD 40 repeat region regulates Apaf 1 self association and procaspase 9 activation, J. Biol. Chem. 273:33489 33494.
Huang DC, Strasser A (2000), BH3 only proteins essential initiators of apoptotic cell death, Cell 103:839 842.
Jiang X, Wang X (2000), Cytochrome C promotes caspase 9 activation by inducing nucleotide binding to Apaf 1, J. Biol. Chem. 275:31199 31203.
Jiang X, Wang X (2004), Cytochrome C mediated apoptosis, Annu. Rev. Biochem. 73:87 106.
Kelekar A, Chang BS, Harlan JE, Fesik SW, Thompson CB (1997), Bad is a BH3 domain containing protein that forms an inactivating dimer with Bcl X_L, Mol. Cell Biol. 17:7040 7046.

Kerr JF, Wyllie AH, Currie AR (1972), Apoptosis: a basic biological phenomenon with wide ranging implications in tissue kinetics, Br. J. Cancer 26:239 257.
Kilic M, Schafer R, Hoppe J, Kagerhuber U (2002), Formation of noncanonical high molecular weight caspase 3 and 6 complexes and activation of caspase 12 during serum starvation induced apoptosis in AKR 2B mouse fibroblasts, Cell Death Differ. 9:125 137.
Kim NS, Lee GM (2002), Response of recombinant Chinese hamster ovary cells to hyperosmotic pressure: effect of Bcl 2 overexpression, J. Biotechnol. 95:237 248.
Krajewski S, Tanaka S, Takayama S, Schibler MJ, Fenton W, Reed JC (1993), Investigation of the subcellular distribution of the Bcl 2 oncoprotein: residence in the nuclear envelope, endoplasmic reticulum, and outer mitochondrial mem branes, Cancer Res. 53:4701 4714.
Kuwana T, Newmeyer DD (2003), Bcl 2 family proteins and the role of mitochondria in apoptosis, Curr. Opin. Cell Biol. 15:691 699.
Kuwana T, Mackey MR, Perkins G, Ellisman MH, Latterich M, Schneiter R, Green DR, Newmeyer DD (2002), Bid, Bax, and lipids cooperate to form supramolecu lar openings in the outer mitochondrial membrane, Cell 111:331 342.
Letai A, Bassik MC, Walensky LD, Sorcinelli MD, Weiler S, Korsmeyer SJ (2002), Distinct BH3 domains either sensitize or activate mitochondrial apoptosis, serving as prototype cancer therapeutics, Cancer Cell 2:183 192.
Li H, Zhu H, Xu C J, Yuan J (1998), Cleavage of BID by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis, Cell 94:491 501.
Li L, Zou L (2005), Sensing, signaling, and responding to DNA damage: organization of the checkpoint pathways in mammalian cells, J. Cell Biochem. 1:298 306.
Loeffler M, Kroemer G (2000), The mitochondrion in cell death control: certainties and incognita, Exp. Cell Res. 256:19 26.
McCarthy NJ (2002), Why be interested in death, In: Jacobson MD, McCarthy NJ (Eds), Apoptosis: The Molecular Biology of Programmed Cell Death, Chapter 1, Oxford University Press, Cambridge.
McCullough KD, Martindale JL, Klotz LO, Aw TY, Holbrook NJ (2001), Gadd153 sensitizes cells to endoplasmic reticulum stress by downregulating Bcl 2 and perturbing the cellular redox state, Mol. Cell Biol. 21:1249 1259.
Martin SJ, Reutelingsperger CPM, McGahon AJ, Rader JA, van Schie RC, LaFace DM, Green DR (1995), Early redistribution of plasma membrane phosphatidyl serine is a general feature of apoptosis regardless of initiating stimulus: inhibition by overexpression of Bcl 2 and Abl, J. Exp. Med. 182:1545 1556.
Marzo I, Brenner C, Zamzami N, Susin SA, Beutner G, Brdiczka D, Remy R, Xie ZH, Reed JC, Kroemer G (1998), The permeability transition pore complex: a target for apoptosis regulation by caspases and bcl 2 related proteins, J. Exp. Med 187:1261 1271.
Mastrangelo AJ, Betenbaugh MJ (1998), Overcoming apoptosis: new methods for improving protein expression systems, Trends Biotechnol. 16:88 95.
Mastrangelo AJ, Hardwick JM, Bex F, Betenbaugh MJ (2000a), Part I. Bcl 2 and Bcl XL limit apoptosis upon infection with alphavirus vectors, Biotechnol. Bioeng. 67:544 554.
Mastrangelo AJ, Hardwick JM, Zou S, Betenbaugh MJ (2000b), Part II. Overexpres sion of bcl 2 family members enhances survival of mammalian cells in response to various culture insults, Biotechnol. Bioeng. 67:555 564.
Mercille S, Massie B (1994a), Induction of apoptosis in nutrient deprived cultures of hybridoma and myeloma cells, Biotechnol. Bioeng. 44:1140 1154.
Mercille S, Massie B (1994b), Induction of apoptosis in oxygen deprived cultures of hybridoma cells, Cytotechnology 15:117 128.
Mercille S, Jolicoeur P, Gervais C, Paquette D, Mosser DD, Massie B (1999), Dose dependent reduction of apoptosis in nutrient limited culture of NS/O myeloma

cells transfected with the E1B 19KDa adenoviral gene, Biotechnol. Bioeng. 63:516 528.
Mitchison JM (2003), Growth during the cell cycle, Int. Rev. Cytol. 226:165 258.
Miyashita T, Reed JC (1995), Tumor suppressor p53 is a direct transcriptional activator of the human bax gene, Cell 80:293 299.
Muzio M, Chinnaiyan AM, Kischkel FC, O'Rourke K, Shevchenko A, Ni J, Scaffidi C, Bretz JD, Zhang M, Gentz R, Mann M, Krammer PH, Peter ME, Dixit VM (1996), FLICE, a novel FADD homologous ICE/CED 3 like protease, is re cruited to the CD95 (Fas/APO 1) death inducing signaling complex, Cell 85:817 827.
Nagata S (2000), Apoptotic DNA fragmentation, Exp. Cell Res. 256:12 18.
Nakano K, Vousden KH (2001), PUMA, a novel proapoptotic gene, is induced by p53, Mol. Cell 7:683 694.
Narita M, Shimizu S, Ito T, Chittenden T, Lutz RJ, Matsuda H, Tsujimoto Y (1998), Bax interacts with the permeability transition pore to induce permeability transi tion and cytochrome c release in isolated mitochondria, Proc. Natl Acad. Sci. U S A 95:14681 14686.
Newbold RF (2002), The significance of telomerase activation and cellular immortali zation in human cancer, Mutagenesis 17:539 550.
Nguyen M, Millar DG, Yong VW, Korsmeyer SJ, Shore GC (1993), Targeting of Bcl 2 to the mitochondrial outer membrane by a COOH terminal signal anchor sequence, J. Biol. Chem. 268:25265 25268.
Nollert MU, Diamond SL, McIntre LV (1991), Hydrodynamic shear stress and mass transport modulation of endothelial cell metabolism, Biotechnol. Bioeng. 38:588 602.
Oda E, Ohki R, Murasawa H, Nemoto J, Shibue T, Yamashita T, Tokino T, Taniguchi T, Tanaka N (2000), Noxa, a BH3 only member of the Bcl 2 family and candidate mediator of p53 induced apoptosis, Science 288:1053 1058.
Oh SKW, Vig P, Chua FKF (1993), Substantial overproduction of antibodies by applying osmotic pressure and sodium butyrate, Biotechnol. Bioeng. 42:601 609.
Oltvai ZN, Korsmeyer SJ (1994), Checkpoints of dueling dimers foil death wishes, Cell 79:189 192.
Oltvai ZN, Milliman CL, Korsmeyer SJ (1993), Bcl 2 heterodimerizes in vivo with a conserved homolog, Bax, that accelerates programmed cell death, Cell 74:609 619.
Ottilie S, Diaz JL, Horne W, Chang J, Wang Y, Wilson G, Chang S, Weeks S, Fritz LC, Oltersdorf T (1997), Dimerization properties of human BAD. Identification of a BH 3 domain and analysis of its binding to mutant BCL 2 and BCL XL proteins, J. Biol. Chem. 272:30866 30872.
Pläsier B, Lloyd DR, Paul GC, Thomas CR, Al Rubeai M (1999), Automatic image analysis for quantification of apoptosis in animal cell culture by annexin V affinity assay, J. Immunol. Methods 229:81 95.
Puthalakath H, Huang DC, O'Reilly LA, King SM, Strasser A (1999), The proapopto tic activity of the Bcl 2 family member Bim is regulated by interaction with the dynein motor complex, Mol. Cell 3:287 296.
Puthalakath H, Villunger A, O'Reilly LA, Beaumont JG, Coultas L, Cheney RE, Huang DC, Strasser A (2001), Bmf: a proapoptotic BH3 only protein regulated by interaction with the myosin V actin motor complex, activated by anoikis, Science 293:1829 1832.
Qin H, Srinivasula SM, Wu G, Fernandes Alnemri T, Alnemri ES, Shi Y (1999), Structural basis of procaspase 9 recruitment by the apoptotic protease activating factor 1, Nature 399:549 557.
Rao RV, Ellerby HM, Bredesen DE (2004), Coupling endoplasmic reticulum stress to the cell death program, Cell Death Differ. 11:372 380.

Saito M, Korsmeyer SJ, Schlesinger PH (2000), BAX dependent transport of cyto chrome c reconstituted in pure liposomes, Nat. Cell Biol. 2:553 555.
Saleh A, Srinivasula SM, Acharya S, Fishel R, Alnemri ES (1999), Cytochrome c and dATP mediated oligomerization of Apaf 1 is a prerequisite for procaspase 9 activation, J. Biol. Chem. 274:17941 17945.
Sanfeliu A, Stephanopoulos G (1999), Effect of glutamine limitation on the death of attached chinese hamster ovary cells, Biotechnol. Bioeng. 64:46 53.
Sattler M, Liang H, Nettesheim D, Meadows RP, Harlan JE, Eberstadt M, Yoon HS, Shuker SB, Chang BS, Minn AJ, Thompson CB, Fesik SW (1997), Structure of Bcl xL Bak peptide complex: recognition between regulators of apoptosis, Science 275:983 986.
Sauerwald TM, Betenbaugh MJ, Oyler GA (2002), Inhibiting apoptosis in mammalian cell culture using the caspase inhibitor XIAP and deletion mutants, Biotechnol. Bioeng. 77:704 716.
Sauerwald TM, Oyler GA, Betenbaugh MJ (2003), Study of caspase inhibitors for limiting death in mammalian cell culture, Biotechnol. Bioeng. 81:329 340.
Schotte P, Van Criekinge W, Van de Craen M, Van Loo G, Desmedt M, Grooten J, Cornelissen M, De Ridder L, Vandekerckhove J, Fiers W, Vandenabeele P, Beyaert R (1998), Cathepsin B mediated activation of the proinflammatory caspase 11, Biochem. Biophys. Res. Commun. 251:379 387.
Shimizu S, Narita M, Tsujimoto Y (1999), Bcl 2 family proteins regulate the release of apoptogenic cytochrome C by the mitochondrial channel VDAC, Nature 399:483 487.
Simpson NH, Milner A, Al Rubeai M (1997), Prevention of hybridoma cell death by bcl 2 during suboptimal culture conditions, Biotechnol. Bioeng. 54:1 16.
Simpson NH, Singh RP, Perani A, Goldenzon C, Al Rubeai M (1998), In hybridoma cultures, deprivation of any single amino acid leads to apoptotic death, which is suppressed by the expression of the bcl 2 gene, Biotechnol. Bioeng. 59:90 98.
Singh RP, Al Rubeai M (1998), Apoptosis and bioprocess technology, Adv. Biochem. Eng. Biotechnol. 62:167 184.
Singh RP, Al Rubeai M, Gregory CD, Emery AN (1994), Cell death in bioreactors: a role for apoptosis, Biotechnol. Bioeng. 44:720 726.
Singh RP, Finka G, Emery AN, Al Rubeai M (1997), Apoptosis and its control in cell culture systems, Cytotechnology 23:87 93.
Slee EA, Harte MT, Kluck RM, Wolf BB, Casiano CA, Newmeyer DD, Wang HG, Reed JC, Nicholson DW, Alnemri ES, Green DR, Martin SJ (1999), Ordering the cytochrome C initiated caspase cascade: hierarchical activation of caspases 2, 3, 6, 7, 8 and 10 in a caspase 9 dependent manner, J. Cell Biol. 144:281 292.
Srinivasula SM, Ahmad M, Fernandes Alnemri T, Alnemri ES (1998), Autoactivation of procaspase 9 by Apaf 1 mediated oligomerization, Mol. Cell 1:949 957.
Suzuki E, Ollis DF (1989), Cell cycle model for antibody production kinetics, Biotechnol. Bioeng. 34:1398 1402.
Suzuki E, Ollis DF (1990), Enhanced antibody production at slowed growth rates: experimental demonstration and a simple structured model, Biotechnol. Prog. 6:231 236.
Szegezdi E, Fitzgerald U, Samali A (2003), Caspase 12 and ER stress mediated apoptosis: the story so far. Ann. N Y Acad. Sci. 1010:186 94.
Tey BT, Al Rubeai M (2004), Suppression of apoptosis in perfusion culture of myeloma NS0 cells enhances cell growth but reduces antibody productivity, Apoptosis 9:843 852.
Thornberry NA, Rano TA, Peterson EP, Rasper DM, Timkey T, Garcia Calvo M, Houtzager VM, Nordstrom PA, Roy S, Vaillancourt JP, Chapman KT, Nicholson DW (1997), A combinatorial approach defines specificities of members of the caspase family and granzyme B. Functional relationships established for key

mediators of apoptosis, J. Biol. Chem. 272:17907 17911.
Tinto A, Gabernet C, Vives J, Prats E, Cairo JJ, Cornudella L, Godia F (2002), The protection of hybridoma cells from apoptosis by caspase inhibition allows culture recovery when exposed to non inducing conditions, J. Biotechnol. 95:205 214.
Verhagen AM, Ekert PG, Pakusch M, Silke J, Connolly LM, Reid GE, Moritz RL, Simpson RJ, Vaux DL (2000), Identification of DIABLO, a mammalian protein that promotes apoptosis by binding to and antagonizing IAP proteins, Cell 102:43 53.
Vives J, Juanola S, Cairo JJ, Gòdia F (2003a), Metabolic engineering of apoptosis in cultured animal cells: implication for the biotechnology industry. Metab. Eng. 5:124 132.
Vives J, Juanola S, Cairo JJ, Prats E, Cornudella L, Gòdia, F (2003b), Protective effect of viral homologues of Bcl 2 on hybridoma cells under apoptosis inducing condition, Biotechnol. Prog. 19:84 89.
Walker NP, Talanian RV, Brady KD, Dang LC, Bump NJ, Ferenz CR, Franklin S, Ghayur T, Hackett MC, Hammill LD (1994), Crystal structure of the cysteine protease interleukin 1 beta converting enzyme: a (p20/p10)2 homodimer, Cell 78:343 352.
Xie S, Xie B, Lee MY, Dai W (2005), Regulation of cell cycle checkpoints by polo like kinases, Oncogene 24:277 286.
Yamin TT, Ayala JM, Miller DK (1996), Activation of the native 45 kDa precursor form of interleukin 1 converting enzyme, J. Biol. Chem. 271:13273 13282.
Yang X, Chang HY, Baltimore D (1998), Autoproteolytic activation of procaspases by oligomerization, Mol. Cell 1:319 325.
Yoneda T, Imaizumi K, Oono K, Yui D, Gomi F, Katayama T, Tohyama M (2001), Activation of caspase 12, an endoplasmic reticulum (ER) resident caspase, through tumor necrosis factor receptor associated factor 2 dependent mechanism in re sponse to the ER stress, J. Biol. Chem. 276:13935 13940.
Zanghi AJ, Fussenegger M, Bailey JE (1999), Serum protects protein free competent Chinese hamster ovary cells against apoptosis induced by nutrient deprivation in batch culture, Biotechnol. Bioeng. 64:108 119.
Zha J, Harada H, Yang E, Jockel J, Korsmeyer SJ (1996), Serine phosphorylation of death agonist BAD in response to survival factor results in binding to 14 3 3 not BCL X(L), Cell 87:619 628.
Zha J, Harada H, Osipov K, Jockel J, Waksman G, Korsmeyer SJ (1997), BH3 domain of BAD is required for heterodimerization with BCL XL and pro apoptotic activity, J. Biol. Chem. 272:24101 24104.
Zhou Q, Krebs JF, Snipas SJ, Price A, Alnemri ES, Tomaselli KJ, Salvesen GS (1998), Interaction of the baculovirus anti apoptotic protein p35 with caspases. Specifi city, kinetics, and characterization of the caspase/35 complex, Biochemistry 37:10757 10765.
Zong WX, Lindsten T, Ross AJ, MacGregor GR, Thompson CB (2001), BH3 only proteins that bind pro survival Bcl 2 family members fail to induce apoptosis in the absence of Bax and Bak, Genes Dev. 15:1481 1486.

8 Mathematical models for growth and product synthesis in animal cell culture

Elisabeth F.P. Augusto, Manuel F. Barral, and Rosane A.M. Piccoli

8.1 Introduction

It is extremely important from a scientific and technological point of view to understand cell behavior under different culture conditions. This knowledge allows control of the processes that generate important commercial products and establishes a rational basis to increase cell productivity and yield.

By understanding cell behavior, it is possible to identify factors that limit and inhibit cell growth and product synthesis. This allows improvements in culture media formulation, defining adequate culture conditions and keeping them at optimum values through process control. The optimization of these conditions allows an increase in reaction rates and their control, resulting in enhanced process productivity, higher final product concentration, and increased substrate-to-product conversion factor. This process optimization, in general, may also allow improvements in product quality, insuring technical and economic viability.

The systematization and quantification of this knowledge through mathematical equations, called mathematical modeling, relate culture conditions to growth, death, nutrient consumption, and product synthesis rates. Solving these equations allows a quantitative prediction of cell behavior, meaning that it is possible to simulate the changes in the major variables (such as component concentrations) with time. Even at the initial stages of model development, before it is capable of representing the totality of the observed phenomena, it is possible to use a model for the simulation of different culture conditions. The capacity to predict the dynamics of the population helps to understand the system under investigation, because it may guide experimental planning, reduce the number of experiments, and allow estimates of un-measured variables, as well as providing rational criteria for process design, optimization, and control of bioreactors.

Although animal cells have the ability to produce high value proteins with post-translational modifications appropriate for clinical application,

there is an urgent need to increase bioprocess efficiency and productivity. The reduction of cost is essential to insure that animal cell culture remains competitive compared to alternative technologies such as ribosome display and transgenic animals. Improvements can be made by a biological approach, as discussed in Chapter 3, or based on the optimization of culture conditions. Mathematical modeling is probably the most efficient technique for the optimization of culture conditions.

The construction of a mathematical model involves the following steps, as developed by several authors (Engasser *et al*., 1998; Miller and Reddy, 1998; Bonomi and Schmidell, 2001).

(i) **Formulation of kinetic equations.** This is probably the most important step in developing models because it establishes mathematical relations between the observed phenomena and the cellular responses. It depends on several tasks, namely:
   - Definition of variables relevant to the process: **state variables**, such as cells, substrates, and product concentrations, that characterize the system studied; and **operational variables**, that represent particular conditions of the system, that may be initial or fixed conditions such as initial concentrations, feeding rates, etc.;
   - Kinetic analysis of experimental data and proposed hypotheses of the process limitations or inhibitory steps, which correspond to the identification of the dominant phenomena in the system;
   - Selection of mathematical equations able to represent the specific rates (for growth, product synthesis and substrate consumption) that can describe the phenomena identified in the previous steps.

(ii) Establishment of **mass balances**, total and for each component, considering the operational and state variables. Examples of mass balances for different operation modes (batch, fed-batch, continuous, etc.) are presented in Chapter 9. Depending on the defined variables in the model, it may be necessary to perform energy and momentum balances.

(iii) **Parameter fitting**. Mathematical model equations contain state variables and one or more parameters that are mathematical constants. This step of model formulation, called parameter fitting, consists in defining optimal values for these parameters that minimize the discrepancies between experimental values and the values calculated through the equations (simulated values).

(iv) **Model validation.** The applicability of a model comes from its capacity to predict cell behavior under different culture conditions. Thus, the procedure for model validation consists of comparative statistical analysis between real values (experimental) and calculated values (model simulation). This is an indispensable step to complete the task of modeling any system.

The crucial step in model building is model formulation, since the mathematical modeling is intended to represent a large network of multiple biochemical reactions, controlled by complex regulatory processes that

cause cell adaptation to changes in culture conditions. The complexity of cell metabolism may be treated by different approaches, and both the type of information needed and the material resources to get it should be taken into account when modeling.

The systematization of biological data through mathematical modeling is not a trivial task and requires the intensive use of material and human resources. Moreover, the models often incorporate experimental uncertainties that do not always allow the identification of the trends of the culture, with the desired precision. *Figure 8.1* summarizes the main types of models applied to the description of cellular metabolism, according to the classification proposed by Tsuchiya *et al.*, (1966).

Unstructured and non-segregated models allow the most simplified representation of cellular complexity. As indicated in *Figure 8.1*, in these models, the entire cell population is represented by only one kind of cell (the average cell). Differences between cells within the population are not considered and thus, the cell population is considered homogeneous.

Although an unstructured and non-segregated model includes simplifications of the cellular complexity, it is often used in culture simulation, because it is an adequate compromise between the available data, the difficulties involved in model formulation, and the desired model precision.

The **unstructured and segregated** model considers the cell culture as a heterogeneous population, with individual cells characterized by age, mass, or size. The variation of properties follows a statistical distribution. The most concise way to deal with such population heterogeneity is through a population balance model (PBM). These models can become mathematically complex because the equations that describe the population have to consider that the properties vary in the population and

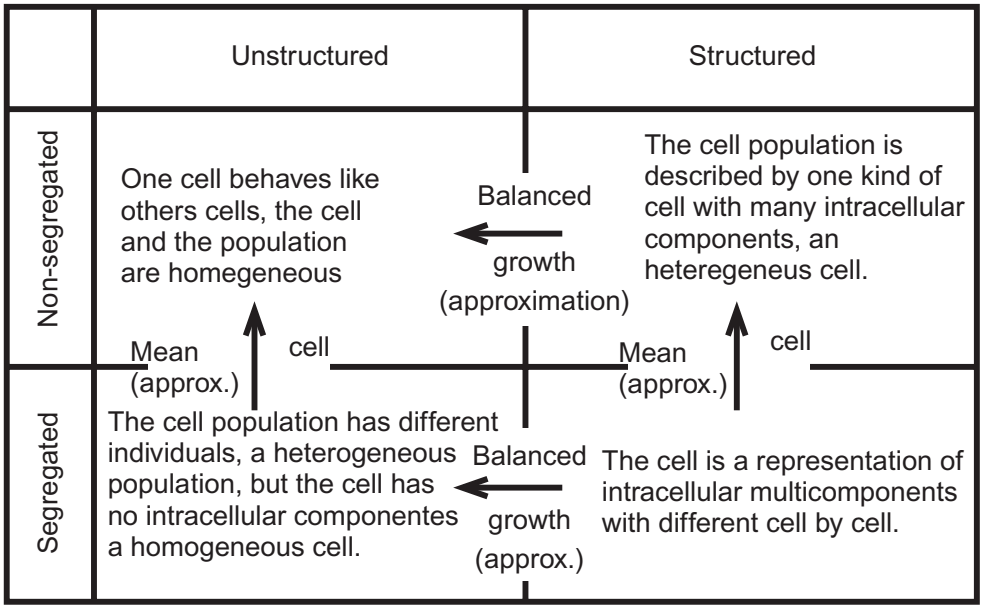


Figure 8.1

Model classification (based on Tsuchiya *et al.*, 1966; Bailey and Ollis, 1986).

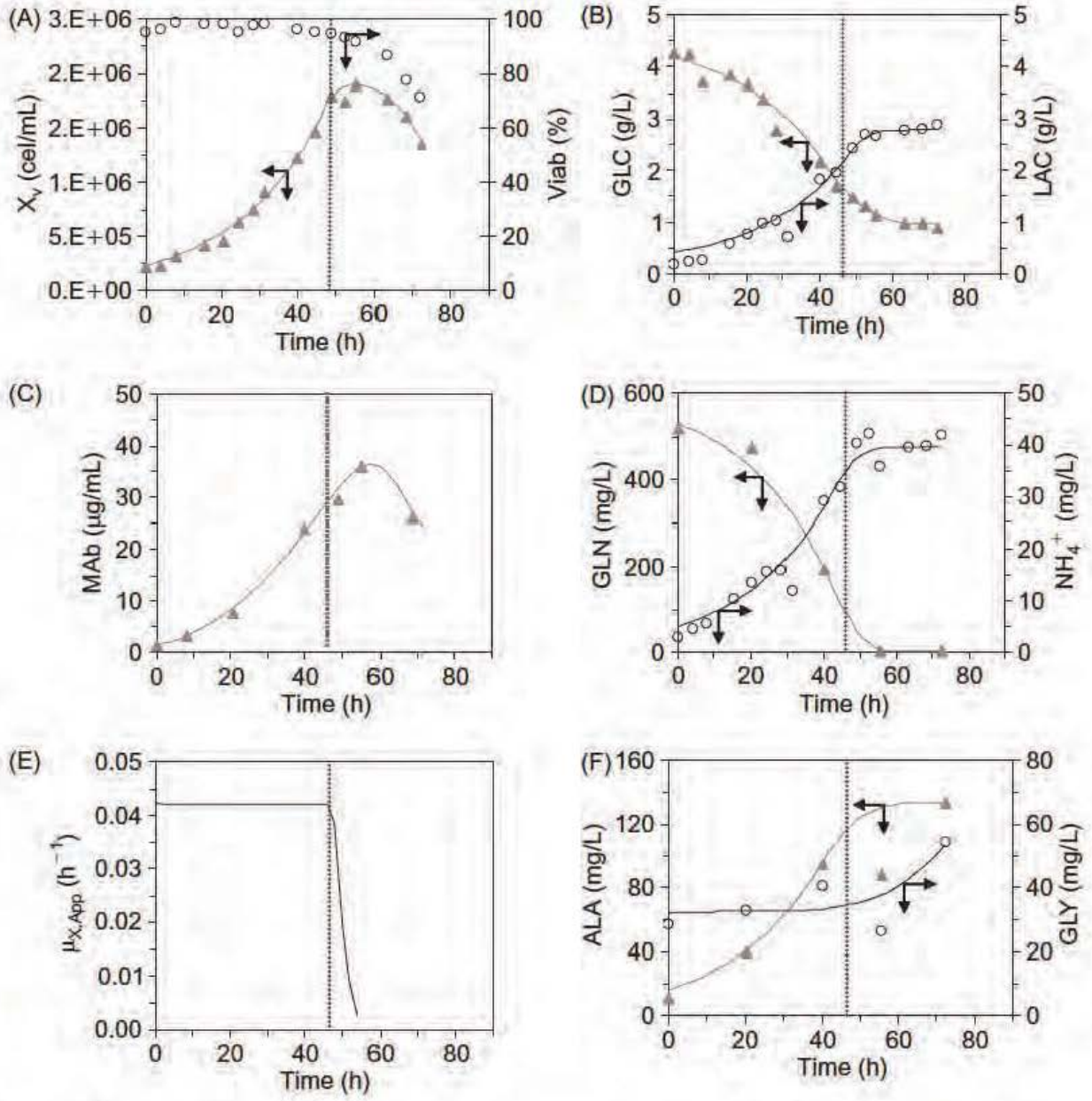


Figure 8.2

Typical kinetic profiles (hybridoma). (A) Cell concentration and viability; (B) glucose consumption (GLC) and lactate production (LAC); (C) monoclonal antibody production (mAb); (D) glutamine consumption (GLN) and ammonium production (NH_4^+); (E) specific growth rate (μ_x); (F) alanine (ALA) and glycine (GLY) production. Adapted from Lee (2003). Symbols correspond to the experimental data and the lines to the manual curve fitting. Vertical lines indicate the instant at which exponential growth phase ended ($\mu_X < \mu_{X,max}$).

during the time course of cell culture. The mathematical description in a segregated model is based on partial differential equations, while the non-segregated model is described entirely by ordinary differential equations. Although this complexity adds difficulties to the mathematical solution of the problem, and also to the estimation of model parameters, new mathematical tools and advances in process monitoring have made possi-

ble the use of such models, changing the past situation when these models were restricted to academic research.

The **structured and non-segregated model** considers the cellular population as homogeneous with reference to certain characteristics (age, size, and mass), however the intracellular structures are discriminated, in such a way that each cell represents a heterogeneous structure. Thus, it is possible to characterize with more precision, the cellular state through the knowledge of the dynamics of these internal structures and its response to the environment conditions. The main advantage of the use of these models is an improved knowledge about the process and, consequently, the capacity to predict cell adaptation to environmental changes. Certainly, increasing the number of cell structures results in an increase of the number of equations and parameters required to describe process dynamics. Moreover, intracellular measurements that require sophisticated methodologies and equipment are necessary. In the past, the use of this type of model was restricted to academic research, but in recent years, it has become more feasible for use in process simulation and evaluation.

The **structured and segregated model** considers a heterogeneous cell population and the cell as a heterogeneous structure, thus representing the diversity of physiological states as well as intracellular structures and the metabolic pathways associated with each structure. This type of model represents the complexity occurring in cell culture. Although these models aim to represent cell growth in a more realistic way, the complexity involved in the mathematical formulation and in fitting of the parameters makes their applicability very restrictive.

Depending on the degree of complexity, these models can be very difficult to solve mathematically. Even with some simplifications, for instance, separating the cells into discrete populations to avoid infinite equations that could be generated considering a continuous population distribution, difficulties in determining the internal characteristic parameters may still remain.

The complexity involved in cell structured and segregated models is increased with the necessity of their integration and can explain why some authors define the **structured and segregated models** as the next challenge for animal cell modeling. These difficulties are reflected by the scarce number of publications dealing with this integration. This advanced research field is beyond the scope of this book.

The mathematical modeling of animal cell processes was reviewed by Tziampazis and Sambanis (1994), Pörtner and Schäfer (1996), and Sidoli *et al.*, (2004). These authors focused on non-structured and non-segregated models, certainly the most abundant models in literature, and also discussed the evolution of the use of other models, mainly the structured and segregated ones.

8.2 Kinetic analysis of bioprocesses

When studying the kinetic behavior of a system, it is necessary to understand how the concentrations of the representative components of the system (cell, substrate, products, and byproducts) vary with time. Thus,

the analysis of the change of these variables with time allows the calculation of some kinetic variables that characterize the system and are the basis for the identification of the controlling phenomena.

The mathematical modeling of animal cell processes has many elements in common with the modeling of microbial systems. In fact, many examples of mathematical modeling discussed in Section 8.3.1 were initially considered for microorganisms and were later adapted to animal cells.

Figure 8.2 (see page 184) shows the typical behavior of animal cells in culture. Cell growth, substrate consumption, and product synthesis profiles are very similar to those presented in Chapters 2, 4, and 9, and will be used to demonstrate the approach to data treatment usually applied to this kind of process.

Considering that animal cells have high nutritional requirements, the culture medium is always a complex formulation to insure the adequate function of catabolism and anabolism (see Chapters 4 and 5). Glucose and glutamine are precursors for biosynthesis and energy generation pathways, and are the most highly consumed substrates. In parallel, lactate and ammonium are synthesized at high rates as byproducts of glucose and glutamine (or other amino acid) metabolism, respectively. There is also the possibility of secretion of amino acids (mainly alanine, glycine, and aspartate) and of commercially attractive products (monoclonal antibodies, mAbs, for example).

8.2.1 Characteristic kinetic variables

The identification of phenomena that explain the behavior of a studied system depends on the analysis of their kinetic data. Normally, this kinetic analysis is performed using characteristic variables calculated from the experimental data. The specific rates and the yield coefficients are the common values used in this task. When cell concentration data are available, cell growth and death rates, as well as cell viability, are the best kinetic variables to characterize the population physiological state. In the absence of this information – as can occur, for example, with immobilized cells – the treatment must be based on substrate consumption or on metabolites production (Miller and Reddy, 1998).

Specific rates

To predict kinetic behavior it is necessary to know the change of the state variables over time. Thus, it is necessary to determine the characteristic rates of the system under transformation. The instantaneous cell growth rate, for example, is defined in Equation 1.

$$r_X = \frac{dX_V}{dt} \tag{1}$$

where:

X_v = viable cell concentration ($M \cdot L^{-3}$ or $Cel \cdot L^{-3}$);
r_X = cellular growth rate ($M \cdot L^{-3} \cdot T$ or $Cel \cdot L^{-3} \cdot T$);
t = time (T).

As indicated above, the units defined for the listed variables are given in the MLT system, except in cases where cell concentration (given for animal cells as numbers) is involved. M represents mass, L length, and T time.

Similar equations can be written for the substrate consumption rate (r_S) and product synthesis rate (r_P):

$$r_S = -\frac{dS}{dt} \tag{2}$$

$$r_P = \frac{dP}{dt} \tag{3}$$

where:

r_S = substrate consumption rate ($M \cdot L^{-3} \cdot T^{-1}$);
r_P = product synthesis rate ($M \cdot L^{-3} \cdot T^{-1}$);
S = substrate concentration ($M \cdot L^{-3}$);
P = product concentration ($M \cdot L^{-3}$).

The instantaneous rates are necessary for the characterization of the cellular activities but may not be sufficient. This occurs because during a specific interval of time, the concentration of cells can vary significantly, and this may affect transformation rates. Therefore the specific growth rate is defined as a characteristic rate of the population. The specific growth rate is calculated from the cellular growth rate divided by the cellular concentration, and can be represented by the Equation 4 (Miller and Reddy, 1998; Hiss, 2001):

$$\mu_X = \frac{1}{X_V} \cdot \frac{dX_V}{dt} = \frac{1}{X_V} \cdot (r_X) \tag{4}$$

where:

μ_X = specific growth rate (T^{-1}).

The specific growth rate can be calculated from cellular concentration data if the cell viability is sufficiently high. When the cell death rate is significant, the value obtained from Equation 4 is in fact an "apparent" specific rate, since the measured values in the laboratory are determined as a balance between growth and death. The apparent rate is related to the true specific growth rate by the following equation:

$$\mu_X^{ap} = \mu_X - k_d \tag{5}$$

where:

μ_X^{ap} = apparent specific growth rate (T^{-1});
k_d = specific cell death rate (T^{-1}).

The specific death rate can be obtained directly from viable and dead cell concentrations (Equation 6).

$$k_d = \frac{1}{X_v} \cdot \frac{dX_d}{dt} \tag{6}$$

where:

X_d = dead cell concentration ($M \cdot L^{-3}$ or $Cel \cdot L^{-3}$).

The methodology normally used for the determination of dead cell concentrations is the dye exclusion method (Freshney, 1994), although this is not adequate in cases in which cellular lysis is significant. In these situations, other methodologies must be adopted to determine the amount of lysed cells, such as, for example, the measure of the lactate dehydrogenase concentration (LDH) in the culture medium (Freshney, 1994; Miller and Reddy, 1998).

It is possible define the specific substrate consumption rate (Equation 7) and specific product synthesis rates (Equation 8) in a similar way to the specific growth rate.

$$\mu_S = \frac{1}{X_V} \cdot \left(-\frac{dS}{dt}\right) = \frac{1}{X_V} \cdot (-r_S) \tag{7}$$

where:

μ_S = specific substrate consumption rate ($M \cdot M^{-1} \cdot T^{-1}$ or $M \cdot Cel^{-1} \cdot T^{-1}$);

$$\mu_P = \frac{1}{X_V} \cdot \frac{dP}{dt} = \frac{1}{X_V} \cdot (r_P) \tag{8}$$

where:

μ_P = specific product synthesis rate ($M \cdot M^{-1} \cdot T^{-1}$ or $M \cdot Cel^{-1} \cdot T^{-1}$).

Yield coefficients

The **yield** coefficients that measure the substrate conversion into cells and substrate conversion into products are also excellent variables for the characterization of the system, as well as to follow the metabolic changes of the culture. The substrates are consumed to form cells and products (or byproducts), and to generate energy. These conversions have characteristic parallel reactions, as substrate consumption (S) leads to many ''products'', that is, cells, products, and byproducts (*Figure 8.3*).

The substrate-to-cells or substrate-to-products yield coefficients can be calculated in an interval of time Δt, as indicated by Equations 9 and 10.

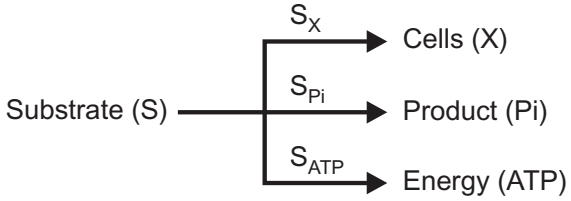


Figure 8.3

Simplified schema of the substrate partition between metabolic pathways; catabolism (used for ATP and ADP synthesis) and anabolism (for biomass synthesis). The quantities S_{Pi} indicate the fraction of S destined to formation of the product ''i''.

$$Y_{X/S} = \frac{\Delta X}{-\Delta S_X} \tag{9}$$

where:
$Y_{X/S}$ = substrate-to-cells yield coefficient (or factor) ($M \cdot M^{-1}$ or $Cel \cdot M^{-1}$);
ΔX = cellular concentration variation in the time interval Δt ($M \cdot L^{-3}$ or $Cel \cdot L^{-3}$);
$-\Delta S_X$ = substrate consumption for cell formation in time interval Δt ($M \cdot L^{-3}$).

$$Y_{P/S} = \frac{\Delta P}{-\Delta S_P} \tag{10}$$

where:
$Y_{P/S}$ = substrate-to-product yield coefficient (or factor) ($M \cdot M^{-1}$);
ΔP = product concentration variation in the time interval Δt ($M \cdot L^{-3}$);
$-\Delta S_P$ = substrate consumption for the product formation in the time interval Δt ($M \cdot L^{-3}$).

If the final destination of the substrate is unique, the yield coefficient (to cell or products) is a stoichiometric constant that relates reagents to ''products.'' However, it is frequent that the substrate forms more than one substance (for example, glucose may be converted to cells and lactate), or that a specific product comes from distinct substrates (for example, lactate formed from glucose and glutamine). In these cases, the calculation of the real conversion factors is only possible when a labeled reagent is used, allowing the identification of the reagent destination in the metabolic pathway. In practice, global yield coefficients are often calculated from total substrate consumption and the total ''products'' formed, as indicated in Equations 9 and 10. Instantaneous yields coefficients $Y_{X/S}$ and $Y_{P/S}$ can be calculated as a relation between substrate consumption rate and ''products'' formation rate, as indicated in Equations 11 and 12.

$$Y_{X/S} = -\frac{r_X}{r_S} = -\frac{\mu_X}{\mu_S} \tag{11}$$

$$Y_{P/S} = -\frac{r_P}{r_S} = -\frac{\mu_P}{\mu_S} \tag{12}$$

Finally, oxygen consumption per unit of substrate (Equation 13) is a factor that helps in understanding the metabolic pathways utilized by the cells and, therefore, it is useful in system analysis. This not exactly a yield coefficient, because it establishes a relation of consumption between two substrates. Similarly, other relations, not exactly yield coefficients (for example, $Y_{LAC/X}$; $Y_{NH3/X}$, etc.), can be calculated and utilized for system characterization.

$$Y_{O_2/S} = -\frac{\mu_{O_2}}{\mu_S} \tag{13}$$

where:
$Y_{O_2/S}$ = oxygen consumption per unit of substrate factor ($M \cdot M^{-1}$);

μ_{O_2} = specific oxygen consumption rate ($M \cdot M^{-1} \cdot T^{-1}$ or $M \cdot Cel^{-1} \cdot T^{-1}$).

8.2.2 Data treatment

The treatment of experimental data is an essential activity to calculate precise kinetic variables in the equations above. The quality of the kinetic analysis, the identification of relevant phenomena and, subsequently, model parameter fitting, are directly dependent on the initial data.

This section discusses treatment of experimental data, especially for conditions where state variables change over time. These are the most difficult data to treat and correspond to cultures from batch, fed-batch, or any continuous transient phase. In continuous steady state, the state variables and rates values do not alter with time, and the rate calculation results from the algebraic equation solution.

The experimental error is also relevant to data treatment, and will also be considered in this section. The extent of the analytic error depends on various factors including the type of methodology adopted, operator training, and inoculum protocols. The errors are relatively high in biological systems: for example, 10–30% for proteins concentration; 7–10% for cells concentration and viability (especially at low viability); 2–5% for liquid chromatography measures (glucose, lactate, amino acids) (Pörtner and Schäfer, 1996; Miller and Reddy, 1998).

The experimental error of measurements from batch processes is normally higher than those obtained from a stationary state in processes conducted in a continuous mode, simply because, in batch, variations in culture conditions occur during the whole process. In a stationary state of a continuous process, the kinetic variables are calculated from state variable average values.

It is always possible to calculate the kinetic variables directly from the experimental data. However, the values of kinetic variables are of higher quality when the errors are attenuated and identified before employing the treatment data methods.

This attenuation can be made by smoothing methods that allow data extrapolation, if the number of experimental points is reduced. The smoothing methods should also allow rate calculation from these points. The most commonly used smoothing techniques of experimental data are presented below.

(i) **Manual smoothing of the experimental data,** which implies tracing an average curve with experimental points based on previous knowledge of the system. Subsequently, the smoothed curve is read, creating a data table (variable = f(time)).

(ii) **Simple mathematical function fitting –** linear, logarithmic, or polynomial with low degree regressions. This can be sufficient to obtain smooth experimental data. By applying the minimal quadratic method, it is possible to fit any of these functions, minimizing the difference between real data and those simulated by functions. This is a relatively easy task, since these functions are available in various commercial software products. Although it is difficult to represent

the entire culture profile by one curve, it is always possible to fit different functions to the various phases of culture (iii and iv below).

(iii) **Mathematical function fitting by stepwise changes**. This approach aims to represent the growth phase by stepwise changes of functions. The "spline" method, for example, consists of a series of low degree polynomial regressions, which fit a low number of experimental points (3 to 4 points) each time, ensuring continuity between the various polynomial functions (Lancaster and Salkauskas, 1986).

(iv) **A mixed approach** combines the smoothing of experimental data, based on previous knowledge of the system (i), and mathematical function fitting (linear regression, logarithmic, polynomial, spline, etc.), as outlined in (ii) and (iii) above.

Once the experimental errors are attenuated, data are prepared for the calculation of kinetic variables. Frequently, it is necessary to determine the rates (Equations 4 to 8), and the possible approaches are:

(v) the derivation of mathematical functions fitted, as proposed in (ii) and (iv) above;

(vi) the calculation of derivatives employing geometrical methods, as Le Duy and Zajic (1973), applied to experimental smoothed data (Hiss, 2001).

The determination of these rates allows the direct calculation of instantaneous yield factors, as in Equations 11 and 12. This form of calculation is employed whenever these factors change during the cultivation time. Alternatively, it is possible to determine yield factors by linear regression of "product" concentration (cell, byproducts, or product) as a function of the substrate concentration.

8.2.3 Phenomena identification

A lot of effort as regards phenomena identification is based on specific growth rate analysis. In Chapter 2, cell growth and division were proposed in three phases, based on values of specific growth rates. The number of steps observed in a culture depends on each system, that is, some steps may not exist or have an insignificant duration under some conditions.

A lag (or latent or adaptation) phase can occur at the beginning of culture during which no growth occurs, followed by an acceleration phase, a period in which the specific growth rate varies until reaching a maximum value. The analysis of this initial behavior can reveal any problems connected with preparation of inocula, as well as any substrate inhibition.

The other typical situation is that shown in *Figure 8.2E*, in which the μ_X is maximum from the beginning of culture. During the exponential growth phase, the specific growth rate remains at the maximum value ($\mu_{X,max}$) until any nutritional limitation or inhibition by toxic products alters cell growth. Thus, a specific growth rate reduction can be observed.

The behavior analysis of the other state variables (substrates and product concentrations), at the same time as the μ_X decrease, can indicate the cause of the metabolism change. For the case exemplified in *Figure 8.2* it can be observed that at 47 hours (end of exponential phase) the

glutamine concentration is very low (*Figure 8.2D*), which can indicate a limitation of this nutrient.

It may be necessary to verify if the lactate levels (~2.2 g/L) and/or ammonium (~35 mg/L) reach inhibitory concentration values. It is necessary to consider the possibility of the occurrence of limitation and inhibitory effects quite frequently in the culture. To discriminate between the influences of several phenomena it is necessary to do tests under conditions specifically planned for this purpose, allowing the investigator to distinguish between limitation and inhibition phenomena.

The rate of change of μ_X at the decline phase can also help in phenomena identification. For example, an abrupt fall in the μ_X value is an indication of nutrient limitation. Once the limiting nutrient has reached a critical concentration, complete depletion and a decrease in growth will occur quickly. On the other hand, when a reduction of μ_X occurs slowly, the phenomenon responsible is usually the formation of an inhibiting metabolite, which has gradually accumulated in the system.

There are other ways to identify the factors that regulate cell metabolism, and each researcher may establish suitable methods for a particular system based on kinetic data analysis (Sinclair and Kristiansen, 1987; Engasser *et al.*, 1998; Bonomi and Schmidell, 2001). Nevertheless, the importance of adequate experimental data treatment is evident. This would allow precise specific rate calculations and identification of the associated phenomena.

8.3 Unstructured and non-segregated models

As stated previously, unstructured and non-segregated models are those most commonly employed for simulating cell culture, due to their simplicity in representing the biological complexity of the system. It is should be restated that these models consider the cell population as homogeneous and do not distinguish between individual cells.

In this section, a classical set of equations – initially developed for microbial systems – will be presented, followed by a review of the most important models for cell culture in the literature.

8.3.1 Classical formulas for cell growth, substrate consumption, and product synthesis

From kinetics studies of unicellular organisms, a set of mathematical expressions have been established to represent the most frequent phenomena in bioprocesses. These phenomena involve a limitation or inhibition of growth and product formation, caused by the presence of substrates, products, or byproducts in culture media. Many of these expressions do not derive from known kinetic mechanisms. In fact, they are simply mathematical expressions with fitted parameters that are able to reproduce experimentally observed kinetic profiles. These equations have been derived and used in many unstructured microbial or cell models.

Cell growth

Substrate limitation

Many of the equations employed in unstructured and non-segregated models derive from those of enzymatic kinetics (Sinclair and Kristiansen, 1987; Nielsen and Nikolajsen, 1988). Cells are considered as chemical reactors that support thousands of complex reactions catalyzed by enzymes that allow the conversion of substrates into secreted products. The equation formulated by Michaelis and Menten represents the enzymatic conversion rate of a unique substrate into one product (Equation 14).

$$r_E = k \cdot E \cdot \frac{S}{k_m + S} \tag{14}$$

where:

r_E = reaction rate;
k = reaction constant;
E = enzyme total amount;
k_m = Michaelis constant;
S = limiting substrate concentration.

The amount (k.E) is the maximum reaction rate ($r_{E,max}$), that always occurs at conditions where limiting substrate concentrations are much higher than the constant k_m ($S >> k_m$), allowing enzyme saturation by substrate.

By analogy to Michaelis and Menten enzymatic kinetics, Monod (1949) proposed the formula shown in Equation 15 that represents the cell growth rate as a function of cell and substrate concentrations.

$$r_X = \mu_{X,max} \frac{S}{k_S + S} \cdot X \tag{15}$$

where:

$\mu_{X,max}$= maximum specific cell growth rate (T^{-1});
k_S = substrate limitation constant for growth (or substrate saturation constant for growth) ($M \cdot L^{-3}$);
S = limiting substrate concentration ($M \cdot L^{-3}$);
X = cell concentration ($M \cdot L^{-3}$ or $Cel \cdot L^{-3}$).

This same equation can be written as a function of the specific growth rate (Equation 16).

$$\mu_X = \mu_{X,max} \frac{S}{k_S + S} \text{ (Monod, 1949)} \tag{16}$$

Thus, the specific growth rate can be expressed by two parameters (constants) – $\mu_{X,max}$ and k_S – and one state variable – S. This allows a parameter fitting to experimental data of cell (X) and substrate (S) concentrations. The procedure to fit these parameters will be shown in Section 8.3.3.

Figure 8.4 presents simulations of the Monod model with different sets of parameters. As a general rule, whenever the limiting substrate is in excess ($S >> k_S$), specific cell growth takes the maximum value ($\mu_{X,max}$), and becomes independent of substrate.

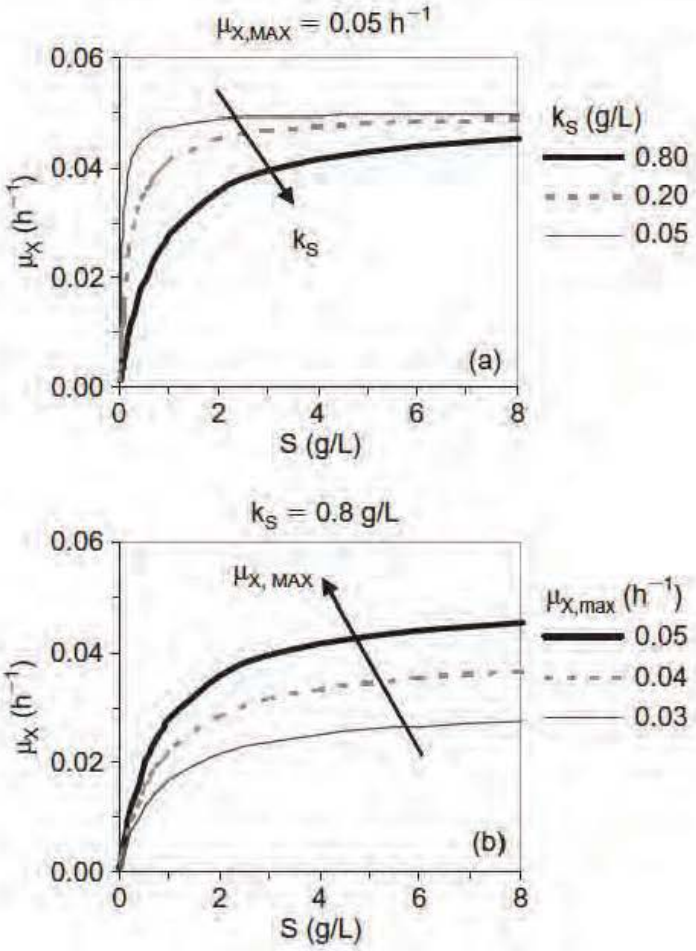


Figure 8.4

Specific growth rate as a function of limiting substrate S, for different sets of parameters $\mu_{X,max}$ and k_S.

When the limiting substrate reaches the concentration equivalent to k_S, the specific growth rate assumes the value of $\frac{\mu_{X,max}}{2}$. This is the critical substrate concentration for cell growth.

Certainly, Monod's formula has been used extensively in phenomenological (unstructured) models, although the literature presents other equations for one limiting substrate systems (Equations 17 and 18). In Moser's formulation it was necessary to introduce a third parameter ("n" in Equation 17) to represent experimental data.

$$\mu_X = \mu_{X,max} \frac{S^n}{k'_S + S^n} \text{ (Moser, 1958)} \tag{17}$$

where:

k'_S = constant $((M \cdot L^{-3})^2)$

$$\mu_X = \mu_{X,max} \frac{S}{k''_S X + S} \text{ (Contois, 1959)} \tag{18}$$

where:
k''_S = constant ($M \cdot M^{-1}$ or $M \cdot Cel^{-1}$)

Equation 19 presents a kinetic model for dual-limiting substrates (S_1 and S_2) in culture. In such cases, the Monod structures for substrate limitation ($\frac{S_i}{k_{S_i}+S_i}$) are reproduced for each one of the limiting substrates.

$$\mu_X = \mu_{X,max} \frac{S_1}{k_{S_1} + S_1} \cdot \frac{S_2}{k_{S_2} + S_2} \text{ (Megee } et\ al.\text{, 1972)} \quad (19)$$

The proposal made by Megee *et al.* (1972) is valid whenever limiting substrates are consumed simultaneously and both are essential for growth. This means that growth is interrupted when complete consumption of any substrate occurs, even though the other substrate is in excess. Dunn *et al.* (1992) proposed the idea of enhancing substrates that are non-essential, but if available they can improve specific cell growth rate (Equation 20).

$$\mu_X = \frac{\mu_{X,max1} \cdot S_1}{k_{S_1} + S_1} + \frac{\mu_{X,max2} \cdot S_2}{k_{S_2} + S_2} \text{ (Dunn } et\ al.\text{, 1992)} \quad (20)$$

Substrate inhibition
Models formulated in Equations 21 and 22 describe the situation of a medium component that can be either a limiting substrate or an inhibiting substrate. At the beginning of a culture, the high substrate concentration causes growth inhibition; nevertheless the gradual consumption by cells reduces this effect until concentration gets low enough to become limiting.

$$\mu_X = \mu_{X,max} \frac{S}{k_S + S + \frac{S^2}{k_{i,S}}} \text{ (Andrews, 1968)} \quad (21)$$

where:
$k_{i,S}$ = substrate inhibition constant for growth ($M \cdot L^{-3}$).

$$\mu_X = \mu_{X,max} \frac{S}{1 + \frac{k_S}{S} + \left(\frac{S}{k'_{i,S}}\right)^n} \text{ (Wu } et\ al.\text{, 1988)} \quad (22)$$

where:
$k'_{i,S}$ = constant ($(M \cdot L^{-3})^n$).

Product inhibition
Equations 23 to 25 are expressions of growth inhibition due to synthesis of byproducts during culture. Those formulas can represent exponential, hyperbolic, and linear reduction profiles of specific growth rates as a function of product accumulation in the culture environment.

$$\mu_X = \mu_{X,max} \cdot e^{-k'_{i,P} \cdot P} \text{ (Aiba } et\ al.\text{, 1968)} \quad (23)$$

where:
$k'_{i.P}$ = product inhibition constant for growth ($L^3 \cdot M^{-1}$).

$$\mu_X = \mu_{X,max} \cdot \frac{k_{i,p}}{k_{i,P} + P} \text{ (Aiba and Shoda, 1969)} \quad (24)$$

where:
$k_{i,P}$ = product inhibition constant for growth ($M \cdot L^{-3}$).

$$\mu_X = \mu_{X,max} \cdot \left(1 - \frac{P}{P_m}\right) \text{ (Ghose and Tyagi, 1979)} \quad (25)$$

where:
P_m = maximum product concentration that states for $\mu_X = 0$ ($M \cdot L^{-3}$).

Substrate consumption

The classical studies of Monod (1942) demonstrated a carbon flux partition between catabolism and anabolism, and suggested that a small amount of the carbon uptake is used for maintenance purposes. However, at that time it was impossible to determine the consumption needed for cell maintenance, because the methodologies lacked precision.

Later, it was observed that substrate to cell yield factors ($Y_{X/S}$) could vary as a function of specific growth rate. Pirt (1966) described a linear relationship between growth and substrate consumption, as well as the statement of a term for cell maintenance (Equation 26).

$$\mu_S = \frac{1}{(Y_{X/S}^{max})} \cdot \mu_X + m_S \text{ (Pirt, 1966)} \quad (26)$$

where:
m_S = specific substrate uptake rate for maintenance, or maintenance coefficient ($M \cdot M^{-1} \cdot T^{-1}$ or $M \cdot Cel^{-1} \cdot T^{-1}$);
$Y_{X/S}^{max}$ = maximum or global substrate-to-cells yield factor ($M \cdot M^{-1}$ or $Cel \cdot M^{-1}$).

This expression establishes a variation of the real yield factor as a function of specific growth rate (Equation 27).

$$\frac{1}{Y_{X/S}} = \left(\frac{1}{Y_{x/s}^{max}}\right) + \left(\frac{m_S}{\mu_X}\right) \quad (27)$$

The maintenance coefficient can explain the non-growth associated substrate consumption for energy production, an energy that supports transmembrane ions gradient, motility, among other functions.

The expression established by Pirt (1966) describes anaerobic systems and carbon-limited cultures. However, it gives a poor representation under substrate excess (Zeng and Deckwer, 1995a, 1995b). Then, Tsai and Lee (1990) built a model to represent the excessive consumption of substrate, under conditions of substrate excess (Equation 28).

$$\mu_S = \mu_S^* + \mu_S^E \text{ (Tsai and Lee, 1990)} \quad (28)$$

where:
μ_S^* = specific substrate uptake rate under substrate limitation conditions

$(M \cdot M^{-1} \cdot T^{-1}$ or $M \cdot Cel^{-1} \cdot T^{-1})$;
μ_S^E= increment or decline of specific substrate uptake rate due to substrate excess $(M \cdot M^{-1} \cdot T^{-1}$ or $M \cdot Cel^{-1} \cdot T^{-1})$.

Zeng and Deckwer (1995a, 1995b) demonstrated the flexibility of the Tsai and Lee (1990) proposition in representing data from microorganisms and animal cells, using the following equations to describe the terms μ_S^* and μ_S^E:

$$\mu_S^* = \frac{\mu_X}{Y_S^{max}} + m_S \quad \text{(with } S < S^*\text{) (Zeng and Deckwer, 1995a)} \qquad (29)$$

$$\mu_S^E = \Delta\mu_S^{max} \frac{S - S^*}{S - S^* + k_S^S} \quad \text{(with } S \geqslant S^*\text{) (Zeng and Deckwer, 1995a)} \qquad (30)$$

where:
$\Delta\mu_S^{max}$ = maximum increment or decline of specific substrate uptake rate due to substrate excess $(M \cdot M^{-1} \cdot T^{-1}$ or $M \cdot Cel^{-1} \cdot T^{-1})$;
S^*= substrate concentration under substrate limitation $(M \cdot L^{-3})$;
$(S - S^*)$ = excess of substrate $(M \cdot L^{-3})$;
k_S^S = substrate limitation constant for substrate uptake $(M \cdot L^{-3})$.

Product formation

Similarly to cell growth, the literature presents a set of expressions relating specific product synthesis and state variables (Bonomi and Schmidell, 2001).

Equation 31 shows the generic model for product synthesis formulated by Luedeking and Piret (1959). In this model, the product synthesis can be associated to growth (term $\alpha\mu_X$), and also in a non-associated form (β). In many situations only one of the phenomena is observed – the synthesis is associated or non-associated – employing only one of the terms of Equation 31. *Figure 8.5* illustrates the kinetics profiles for the three different possibilities of product synthesis described above.

$$\mu_P = \alpha\mu_X + \beta \text{ (Luedeking and Piret, 1959)} \qquad (31)$$

where:
α = constant for growth-associated production $(M \cdot M^{-1}$ or $M \cdot Cel^{-1})$;
β = constant for non-growth-associated production $(M \cdot M^{-1} \cdot T^{-1}$ or $M \cdot Cel^{-1} \cdot T^{-1})$.

A usual modification for this equation considers the influence of substrate concentration on the non-associated term, usually expressed as a Monod-type limitation (Equation 32).

$$\mu_P = \alpha\mu_X + \frac{\beta_m S}{k_S^p + S} \qquad (32)$$

where:
β_m = constant $(M \cdot M^{-1} \cdot T^{-1}$ or $M \cdot Cel^{-1} \cdot T^{-1})$;
k_S^p = substrate limitation constant for product synthesis $(M \cdot L^{-3})$.

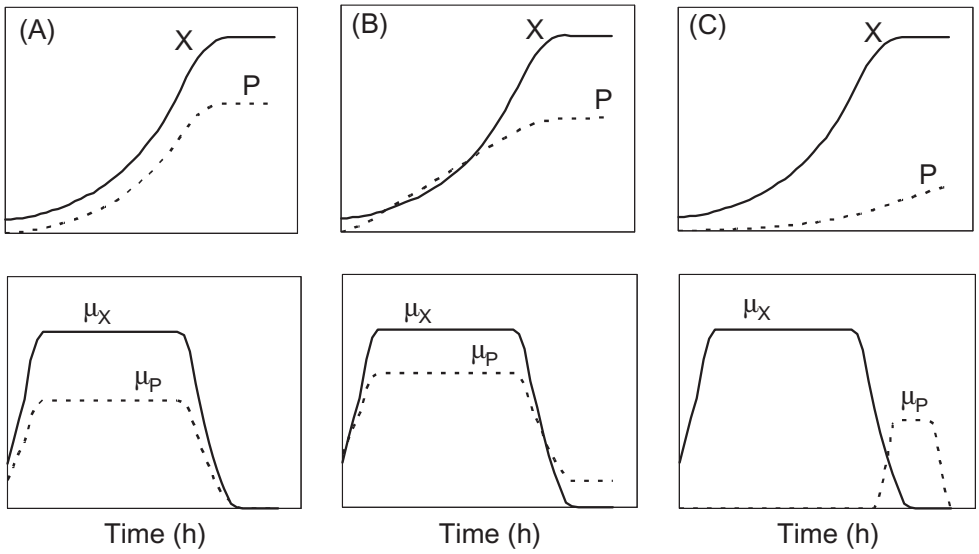


Figure 8.5

Profile for growth and product synthesis according to different production models: (A) growth associated production; B) growth partially associated production, and; C) non growth associated production.

Another form of inhibition of product synthesis results from the product accumulation in the medium itself, that can be modeled using the same structures seen before (Equations 23 to 25) for cell growth inhibition (Equations 33 to 35).

$$\mu_P = \mu_{P,max} \cdot e^{k_{i,P}^{P'} \cdot P} \text{ (Aiba } et\ al.\text{, 1968)} \quad (33)$$

where:

$\mu_{P,max}$ = maximum specific product synthesis rate ($M \cdot M^{-1} \cdot T^{-1}$ or $M \cdot Cel^{-1} \cdot T^{-1}$);

$k_{i,P}^{P'}$ = product inhibition constant for product synthesis ($L^3 \cdot M^{-1}$).

$$\mu_P = \mu_{P,max} \cdot \frac{k_{i,P}^{P}}{k_{i,P}^{P} + P} \text{ (Aiba and Shoda , 1969)} \quad (34)$$

$k_{i,P}^{P}$ = product inhibition constant for product synthesis ($M. \cdot L^{-3}$).

$$\mu_P = \mu_{P,max} \cdot \left(1 - \frac{P}{P'_m}\right) \text{ (Ghose and Thiagi, 1979)} \quad (35)$$

where:

P'_m = maximum product concentration that determines $\mu_P = 0$ ($M \cdot L^{-3}$).

Similarly to what was seen in Equations 28 to 30, Zeng (1995) and Zeng and Deckwer (1995a) proposed a model for specific production rate considering three aspects: production associated with growth; production not associated with growth, and a term due to substrate excess that causes an increment or decline of the specific production rate (Equation 36).

$$\mu_P = \underbrace{Y^*_{P/X} \times \mu_X + m_P}_{\text{Limitation of Substrate}} + \underbrace{\Delta\mu_P^{max} \frac{S\text{-}S^P}{S\text{-}S^P + k_S^P}}_{\text{Excess of Substrate}} \quad (36)$$

(Zeng, 1995; Zeng and Deckwer, 1995a)

where:

$Y^*_{P/X} \cdot \mu_X$ = growth-associated specific production rate, under substrate-limiting conditions;

$Y^*_{P/X}$ = yield coefficient for product formation due to cell growth ($M \cdot M^{-1}$ or $M \cdot Cel^{-1}$);

m_P = nongrowth-associated specific production rate, under substrate-limiting conditions ($M \cdot M^{-1} \cdot T^{-1}$ or $M \cdot Cel^{-1} \cdot T^{-1}$);

$\Delta\mu_P^{max} \frac{S}{S} \frac{S^P}{S^{Pi}+k_S^P}$ = increment or decline of specific production rate due to substrate excess;

$\Delta\mu_P^{max}$ = maximum increment or decline of specific production rate due to substrate excess ($M \cdot M^{-1} \cdot T^{-1}$ or $M \cdot Cel^{-1} \cdot T^{-1}$);

S^P = substrate concentration at which an increase or decrease of μ_P starts to take place ($M \cdot L^{-3}$);

$(S - S^P)$ = substrate excess for product synthesis ($M \cdot L^{-3}$);

k_S^P = substrate limitation constant for product synthesis ($M \cdot L^{-3}$).

8.3.2 Kinetic models for animal cells

In the previous section, the main equations used for building unstructured and non-segregated generic models were discussed. In principle, the description of an animal cell system can be based on any of these formulas, or combinations of them, as can be seen in some literature reviews (Tziampazis and Sambanis, 1994; Pörtner and Schäfer, 1996; Sidoli *et al.*, 2004). Nevertheless, the complexity of animal cell systems also demands alternative mathematical expressions for the full description of observed phenomena.

The identification of such phenomena, as the basis for any mathematical model building, assumes certain knowledge about cell metabolism, under different culture conditions. Chapter 4 discusses the metabolism of animal cells, and should be consulted for a full understanding of the kinetic models presented below.

Kinetics models for cell growth and death, as well as for substrate consumption and product and byproduct synthesis, are presented here. Most of these were developed for hybridomas in continuous processes. Although these models are representative of animal cell systems, it is important to understand that the cellular response to an environmental stimulus is highly dependent on the specific cell line. The review published by Pörter and Schäfer (1996) illustrated this variability through the comparison of experimental data and models from different groups of cell lines. Besides this, the lack of proper knowledge to explain experimentally observed phenomena also accounts for the variability of model structures.

Kinetic formulations for animal cell growth

Table 8.1 summarizes a set of mathematical expressions for the description of the specific growth rate during culture. Most of these formulations employ Monod-type structures for cell growth limitation by substrates (Monod, 1949), and structures for byproduct inhibition (Aiba and Shoda, 1969; see Equations 16 and 24).

Glucose and glutamine, which provide carbon for catabolic and anabolic pathways, appear in the majority of models as limiting substrates. Certainly, the frequency of glucose and glutamine as limiting substrate indicates their importance to cell metabolism, but also the need for

Table 8.1 Kinetic equations for specific hybridoma growth rates

Formulations	References	Eq.
$\mu_X = \mu_{X,max} \cdot \frac{GLN}{k_{GLN} + GLN}$	Pörtner *et al.*, 1996	(37)
$\mu_X = \mu_{X,max} \frac{GLC}{k_{GLC} + GLC} \cdot \frac{GLN}{k_{GLN} + GLN}$	de Tremblay *et al.*, 1992	(38)
$\mu_X = \mu_{X,max} \frac{GLC}{k_{GLC} + Glc} \cdot \frac{k_{i,LAC}}{k_{i,LAC} + LAC}$	Kurokawa *et al.*, 1994	(39)
$\mu_X = \mu_{X,max} \frac{GLN}{k_{GLN} + GLN} \cdot \frac{k_{i,NH_3}}{k_{i,NH3} + NH_3} \cdot \frac{k_{i,LAC}}{k_{i,LAC} + LAC}$	Bree *et al.*, 1988	(40)
$\mu_X = \mu_{X,max} \cdot \frac{GLC}{k_{GLC} + GLC} \cdot \frac{GLN}{k_{GLN} + GLN} \cdot \frac{k_{i,NH_3}}{k_{i,NH_3} + NH_3} \cdot \frac{k_{i,LAC}}{k_{i,LAC} + LAC}$	Miller *et al.*, 1988	(41)
$\mu_X = \mu_{X,min} + (\mu_{X,max} \quad \mu_{X,min}) \cdot \frac{(GLC \quad GLC_{thres})}{k_{GLC} + (GLC \quad GLC_{thres})}$	Frame and Hu, 1991a	(42)
$\mu_X = \mu_{X,max} \cdot (Serum) \cdot \frac{GLC}{k_{GLC} + GLC}$	Dalili *et al.*, 1990	(43)
$\mu_X = \mu_{X,max} \cdot \frac{Serum}{(Serum + (k_{Serum})_0 \cdot X^{-\beta^*})} \cdot \frac{GLN}{k_{GLN} + GLN} \cdot \frac{k'_{i,NH_3}}{k'_{i,NH_3} + (NH_3)^2}$	Glacken *et al.*, 1989	(44)
$\mu_X = a_1 \cdot \frac{B \quad a_2}{B}$	Gaertner and Dhurjati, 1993	(45)
$\mu_X = D + d_0 \cdot e^{(d_1/\mu_X)}$	Linardos *et al.*, 1991	(46)
$\mu_X = \mu_{X,max} \cdot \left(1 \quad \alpha_1 \cdot \frac{X_V}{D}\right) \cdot \frac{GLC}{k_{GLC} + GLC} \cdot \frac{GLN}{k_{GLN} + GLN}$	Zeng *et al.*, 1998	(47)

Adapted from Pörtner and Schäfer, 1996. GLC, glucose; GLN, glutamine; NH_3, ammonia; LAC, lactate; B, base medium concentration; D, specific feed rate; Xv, concentration measured as viable cells number per volume (10^6 cell/ml).

facilities to determine these substances in comparison to other media components. Nevertheless, not all formulas indicate dual limiting-substrate kinetics (GLC and GLN), mainly because the cell response depends upon the cell line and on the culture conditions.

It is possible to represent a kinetic limitation by introducing a threshold concentration, as GLC_{thres} in Equation 42 (Frame and Hu, 1991a). This parameter suggests the existence of an unknown component in culture medium, which causes growth limitation when the glucose concentration reaches the value GLC_{thres}.

Zeng *et al*. (1998) proposed another way to deal with unknown limiting or inhibiting components. From an evaluation of growth, death, and production kinetics of hybridomas in perfusion culture, they noted that neither glucose and glutamine limitation, nor lactate and ammonia inhibition, the most frequent phenomena in an unstructured model, could explain their data. The introduction of parameter α_1 (Equation 47), which relates the specific autoinhibitor production rate and its critical concentration, could represent the behavior of many different cell lines. However, this model shows a strong dependence on the substrate availability, represented by the specific feed rate (D), as well as on viable cell concentration (X_V). This alternative model shows the difficulty, still present, in establishing a cause–effect relationship between a change in the environment and cell behavior.

Gaertner and Dhurjati (1993) used the initial concentration of base medium (B) in an attempt to handle the absence of information concerning the limiting substrate (Equation 45). The base medium corresponded to a DMEM formulation without GLC, GLN, NaCl, and $NaHCO_3$. Different concentrations of this base were tested. The solution showed a hyperbolic relationship between the specific growth rate and the basal medium concentration, independently of the effective limiting component.

Finally, Equations 43 and 44 introduce serum concentration, measured as a percentage of total volume, as the growth-limiting factor. Except for the work of Kurokawa *et al*. (1994), all models presented in *Table 8.1* derive from data obtained in serum-based cultures. However, only two of these models (Glacken *et al.*, 1989; Dalili *et al.*, 1990) utilize this strategy to represent the unknown limiting component.

In general, glucose and glutamine are consumed at a high rate, since cells in culture cannot regulate their uptake. Therefore, cells synthesize large amounts of lactate and ammonia, eventually accompanied by amino acids secretion (alanine, glycine, and aspartate). This absence of regulation causes the rapid depletion of substrates (GLC and GLN) from media, and the consequent accumulation of byproducts (LAC and NH_3), potential inhibitors of the system, both represented in Equations 39 to 41 and 44.

Among the possibilities presented in Section 8.3.1 (Equations 23 to 25), the structure for a hyperbolic inhibitory profile is the most widely used (see Equations 39 to 41, 44 and 47). However, not every formula includes an expression for the potential inhibition of byproducts (LAC and NH_3), since the response is cell-dependent.

The values of the parameters shown in *Table 8.1*, used in building of models, were fitted from different sets of experimental data. Except for very robust models that can represent a large number of situations, differ-

Table 8.2 Fitted parameters for models in Table 8.1

Eq.	$\mu_{X,max}$ (h^{-1})	k_{GLC} (mM)	k_{GLN} (mM)	$k_{i,LAC}$ (mM)	$k_{i,NH3}$ (mM)	Other parameters
(37)	0.036		0.06			
(38)	0.045	1	0.30			
(39)	0.033	0.278		13.9		
(40)	0.125		0.8	8.0	1.05	
(41)	0.063	0.15	0.5	140	20	
(42)			0.0309			$\mu_{X,min} = 0.013\ h^{-1}$; $GLC_{Limiar} = 0.0303$ mM
(43)	0.056	0.06				
(44)	0.055		0.15			$(k_{Serum})_0 = 26.5$; $\beta = 0.21$; $k'_{i,NH_3} = 26\ mM^2$
(45)						$a_1 = 0.043\ h^{-1}$; $a_2 = 0.07$ g/L
(46)						$d_0 = 0.0029\ h^{-1}$; $d_1 = 0.0195\ h^{-1}$;
(47)	0.045 to 0.072	0 to 0.0094	0 to 0.040			$0.0043 \leqslant \alpha_1 \leqslant 0.0092$ ($L/10^9$ cel.h)

ent cell lines and experimental conditions (operation mode, media formulation, initial conditions, etc.) may influence the fitting of parameters and, consequently, different parameter values are expected for the same phenomena. *Table 8.2* presents parameter values fitted for models of *Table 8.1* from specific experimental conditions. These are indicative of the range of values they can assume for animal cell systems. However, some of these values may be considered inappropriate. This is the case of the value fitted for $\mu_{X,max}$ in Equation 40 – 0.125 h^{1} – surely a value not representative for an animal cell system.

Kinetic formulations for animal cell death

Necrosis and apoptosis, the two mechanisms of cell death, are discussed extensively in Chapter 7, and are considered in more detail in this section.

Cell death is particularly important for animal cell systems, considering their intrinsic fragility in the imposed culture conditions, mainly as a result of hydrodynamics shear stress and media composition. This scenario is very different from that of microbial systems, for which it is rare to find formulas representing death processes. When establishing culture conditions for animal cells, there is always much concern in minimizing injury to cells, to insure high viabilities for long periods. Nevertheless, modifications in the environment, mainly in media composition, occur during the culture that normally lead to loss of viability. That explains why many

models incorporate the influence of substrates and products in their equations to simulate death kinetics (*Table 8.3*).

Similar to what has been shown in *Table 8.1* for specific growth rate, many mathematical expressions listed in *Table 8.3* employ Monod-type structures for limiting phenomena, and Aiba and Shoda-type structures for inhibitory behavior. Limiting components for cell death are lactate and ammonia, that is, the presence of these byproducts increases the specific cell death rate. On the other hand, substrates, such as glucose and glutamine, inhibit cell death (Equations 48 to 52).

Similar to what was shown for growth, some models establish a linear relationship between the specific death rate (k_d) and an autoinhibitory product synthesis (Lee *et al.*, 1995). This autoinhibitory product is represented by the expression X_v/D, where X_v is a viable cells concentration, measured in terms of cell number per volume, and D is the specific feed rate that plays the part of substrate supply to the culture. By setting k_d/μ_X as a function of X_t/D (where X_t is a total cell concentration) it is possible to build up a more robust model that can fit a larger amount of experimental data (Equation 56) (Zeng *et al.*, 1998).

Table 8.3 Kinetic expressions for specific cell death rate in animal cell systems

Formulations	References	Eq.
$k_d = k_{d,max} \cdot \frac{NH_3}{k^d_{NH_3} + NH_3} \cdot \frac{LAC}{k^d_{LAC} + LAC}$	Batt and Kompala, 1989	(48)
$k_d = k_{d,max} \cdot \frac{NH_3}{k^d_{NH_3} + NH_3} \cdot \frac{LAC}{k^d_{LAC} + LAC} \cdot \frac{k^d_{i,GLN}}{k^d_{i,GLN} + GLN}$	Bree *et al.*, 1988	(49)
$k_d = \frac{k_{d,max}}{(\mu_{X,max} \quad k^{d*}_{LAC} \cdot LAC) \cdot (\mu_{X,max} \quad k^{d*}_{NH_3} \cdot NH_3)} \cdot \frac{k^d_{i,GLN}}{k^d_{i,GLN} + GLN}$	de Tremblay *et al.*, 1992	(50)
$k_d = k_{d,min} + (k_{d,max} \quad k_{d,min}) \cdot \frac{k^d_{i,GLN}}{k^d_{i,GLN} + GLN}$	Dalili *et al.*, 1990	(51)
$k_d = (\mu_{X,min} \quad D_{min}) \quad k_{d,max} \cdot \frac{(GLC \quad GLC_{thres})}{k^d_{GLC} + (GLC \quad GLC_{thres})}$	Frame and Hu, 1991a.	(52)
$k_d = b_1 + \frac{b_2}{GLN + b_3}$	Pörtner *et al.*, 1996	(53)
$k_d = c_1 \cdot e^{c_2 \cdot \mu_X}$	Glacken *et al.*, 1989	(54)
$k_d = d_0 \cdot e^{(d_1/\mu_X)}$	Linardos *et al.*, 1991	(55)
$k_d = (\beta_0 + \beta_1 \cdot \mu_X) \cdot \frac{X_t}{D}$	Zeng *et al.*, 1998	(56)

Adapted from Pörtner and Schäfer, 1996). Xt, total cell concentration measured as total cell number per volume (10^6 cell/ml).

Table 8.4 lists parameter values fitted to models presented in *Table 8.3*.

Formulations for substrate uptake

Generally, the expressions proposed for the kinetics of glucose or glutamine uptake do not differ significantly from each other. *Table 8.5* (for formulations) and *Table 8.6* (parameters) sum up the kinetic models for the description of substrate uptake rates.

Pirt's formulations, or variations of them (see Equation 26), were employed in many models shown in *Table 8.5*. In one of them (Equation 59) the term $-e^{k_d}$ is added to better fit data obtained at low specific growth rate (Linardos *et al*., 1991). Other propositions introduced a minimum specific growth rate ($\mu_{X,min}$) to fit the non-linearity observed at the same low specific growth rate (Frame and Hu, 1991a, 1991b).

For Equations 62 and 63, the maintenance coefficient appears limited by substrate through a Monod-type formulation.

Finally, in Equation 64, Zeng (1996a) modified his own original model (presented in Equations 28 to 30), by eliminating parameter S* that assumes low values for hybridomas culture. The influence of nutrients is dependent, not on nutrient concentration, but on available mass of nutrient per cell unit S/X_v.

Formulations for product synthesis

Table 8.7 (mathematical expressions) and *Table 8.8* (parameters) review the main models found in the literature for the representation of product

Table 8.4 Fitted parameters for models of Table 8.3

Eq.	$k_{d,max}$ (h^{-1})	$k^d_{NH_3}$ (mM)	k^d_{LAC} (mM)	$k^d_{i,GLN}$ (mM)	Other parameters
(48)	0.08	1.44	311		
(49)	0.0833	1.44	15	5.10^{-4}	
(50)	0.0288			0.02	$k^{d*}_{NH_3} = 0.0025$ (1/mM.h); $k^{d*}_{LAC} = 0.00042$ (1/mM.h)
(51)	0.08			5.10^{-6}	$k_{d,min} = 0.05\ h^{-1}$
(52)	0.00628				$D_{min} = 0.00636\ h^{-1}$; $\mu_{min} = 0.013\ h^{-1}$; $k^d_{GLC} = 0.240$ mM
(53)					$b_1 = 0.002\ h^{-1}$; $b_2 = 6.10^5$ g/L.h; $b_3 = 0.0025$ g/L
(54)	0.051				$c_1 = 0.051\ h^{-1}$; $c_2 = 101.2$ h
(55)					$d_0 = 0.0029\ h^{-1}$; $d_1 = 0.0195\ h^{-1}$
(56)					$0.0 \leqslant \beta_0 \leqslant 4.6.10^{-5}\ h^{-1}$; $0.0 \leqslant \beta_1 \leqslant 0.0038$ L/10^9.cel.h

Table 8.5 Kinetic parameters for specific uptake rates in hybridoma systems: equations are valid for both substrates – GLC and GLN

Formulations	References	Eq.
$\mu_S = \frac{1}{(Y_{X/S}^{max})} \cdot \mu_X$	de Tremblay *et al.*, 1992; Hiller *et al.*, 1991	(57)
$\mu_S = \frac{1}{(Y_{X/S}^{max})} \cdot \mu_X + m_S$	Harigae *et al.*, 1994; Hiller *et al.*, 1991; Miller *et al.*, 1988; Kurokawa *et al.*, 1994	(58)*
$\mu_S = \frac{1}{(Y_{X/S}^{max})} \cdot \mu_X + m_S \quad e^{k_d}$	Linardos *et al.*, 1991	(59)
$\mu_S = \frac{1}{(Y_{X/S}^{max})} \cdot (\mu_X \quad \mu_{min})$	Frame and Hu, 1991a	(60)
$\mu_S = \frac{1}{\frac{1}{Y_{X/S}} \quad \frac{\lambda}{Y_{P/S}}} \cdot \mu_X + \frac{\alpha_2}{Y_{P/S}} \quad \frac{1}{Y_{X/S}} \cdot \mu_{X,min}$	Frame and Hu, 1991b	(61)
$\mu_S = \frac{1}{(Y_{X/S}^{max})} \cdot \mu_X + m_S \frac{S}{k_S^S + S}$	de Tremblay *et al.*, 1992	(62)
$\mu_S = \frac{h_1 \cdot S}{h_2 + S}$	Pörtner *et al.*, 1996; Gaertner and Dhurjati, 1993	(63)
$\mu_S = \left(\frac{1}{Y_S^{max}} \cdot \mu_X + m_S\right) + \Delta\mu_S^m \cdot \frac{S}{S + (X_v \cdot k_S^{S\#})}$	Zeng, 1996a	(64)

*Same formulation proposed in Equation 26.

(proteins) synthesis. Once more, the diversity of mathematical structures reflects the variability of response in such systems, as well as the lack of knowledge of intracellular mechanisms.

Some models utilize the concept of Luedeking and Piret (1959) for growth associated and/or non-growth associated production (Equations 65 to 67). However, in some circumstances, the best fit occurs when the specific death rate k_d is considered, instead of the specific growth rate μ_X (Equations 68, 70 and 71) (Linardos *et al*., 1991; Zeng, 1996a, 1996b). This statement follows the observation that antibody production increases when cells are under conditions of stress.

Considering the influence of media components on specific production rate, only one expression makes serum relevant (Equation 69), while glutamine appears as a limiting substrate in three formulas (Equations 69 to 71), always with a Monod-type structure. Glucose shows either an inhibitory effect over production (Equations 71 and 72), or a limiting and inhibitory pattern simultaneously (Equations 70). Instead of the classical

Table 8.6 Fitted parameters for models of Table 8.5

Eq.	References	S	$Y_{X/S}^{max}$ (10^8 ce /mmo)	m_S (10^{-10} mmo /ce .h)	Other parameters
(57)	de Tremb ay *et al.*, 1992	GLN	3.80	–	–
(58)	Hi er *et al.*, 1991	GLC	1.02	–	
	Mi er *et al.*, 1988	GLC	2.8	0.50	–
	Harigae *et al.*, 1994	GLC	5.88	0.51	–
		GLN	3.44	0.08	–
	Hi er *et al.*, 1991	GLN	16.9	1.56	
(59)	Linardos *et al.*, 1991	GLC	5.93	1.96	–
		GLN	6.30	0.29	–
(60)	Frame and Hu, 1991a	GLC	0.355 mg ce /mg GLC ($p/\mu<0.0508$ h^{-1}): 0.101 mg ce /mg GLC ($p/\mu>0.0508$ h^{-1})		$p/\mu<0.0508$ h^{-1}: $\mu_{min}= 0.00630$ h^{-1} or $p/\mu>0.0508$ h^{-1}: $\mu_{min}= 0.391$ h^{-1}
(61)	Frame and Hu, 1991b	GLC	1.82 ($p/\mu<0.00429$ h^{-1}) 0.52 ($p/\mu>0.00429$ h^{-1})	–	$\alpha_2 = 0.00672$ mg/ce .h; $\lambda = 9.31.10^{-9}$ mg/ce ; $\mu_{X,min} = 0.013$ h^{-1}; $Y_{P/S} = 73.4$ mg/mM
(62)	de Tremb ay *et al.*, 1992	GLC	1.09	0.00708	$k_S^S= 1.0$ mM
(63)	Pörtner *et al.*, 1996	GLN	–	–	$h_1 = 0.68\ 10^{-10}$ mmo /ce .h $h_2 = 0.2$ mmo
	Gaertner and Dhurjati, 1993	GLC	–	–	$h_1 = 2.70\ 10^{-10}$ mmo /ce .h $h_2 = 0.34$ mmo
(64)	Zeng, 1996a	GLC	1.72	0.086	$\Delta\mu_{GLC}^m= 0.094$ L/10^9ce .h $k_{GLC}^s= 0.29$ g/ce
		GLN	9.52	0.0093	$\Delta\mu_{GLN}^m= 0.033$ L/10^9ce .h $k_{GLN}^{S\#}= 0.089$ g/ce

Table 8.7 Kinetic expressions for specific monoclonal synthesis rate

Formulations	References	Eq.
$\mu_{MAb} = \beta$	Pörtner *et al.*, 1996; Hiller *et al.*, 1991	(65)
$\mu_{MAb} = \alpha \cdot \mu_x + \beta$	Frame and Hu, 1991b	(66)
$\mu_{MAb} = \frac{\alpha_0}{k_\mu + \mu_X} \cdot \mu_X + \beta$	de Tremblay *et al.*, 1992	(67)
$\mu_{MAb} = \alpha_1 \cdot k_d + \beta_1$	Linardos *et al.*, 1991	(68)
$\mu_{MAb} = \beta_2 \cdot (\text{Serum}) \frac{GLN}{k_{GLN}^{MAb} + GLN}$	Dalili *et al.*, 1990	(69)
$\mu_{MAb} = (\alpha_1 \cdot k_d + \beta_1) \frac{GLN}{k_{GLN}^{MAb} + GLN} \cdot \frac{k_{i,GLC}^{Mab}}{GLC} \frac{}{GLC^* + k_{i,GLC}^{MAb}} (F_1 + e^{-F_2 \cdot \Delta t})$	Zeng, 1996b	(70)
$\mu_{MAb} = (\alpha_1 \cdot k_d + \beta_1) \cdot \frac{GLN}{k_{GLN}^{MAb\#} \cdot X_v + GLN} \cdot \frac{k_{i,GLC}^{MAb\#} \cdot N_v}{GLC + k_{i,GLC}^{MAb\#} \cdot X_v}$	Zeng, 1996a	(71)
$\mu_{MAb} = \delta \cdot \frac{D_{Per}}{D_{Per} + k_{D_{Per}}^{MAb}} \cdot \frac{k_{i,GLC}^{MAb}}{GLC + k_{i,GLC}^{MAb}}$	Zeng, 1996a	(72)

D_{Per} specific perfusion rate (h^{-1}).

formulation for the limitation or inhibition of a substrate (Equations 21 and 22), a hyperbolic structure is employed to represent inhibition, and the parameter GLC^* is introduced to represent the minimum glucose concentration that guarantees product synthesis.

In Equation 70, Zeng (1996b) considers also the loss of production capacity within the cell population – in the term $(F_1 + e^{F_2 \cdot \Delta t})$. This is a characteristic common to many hybridomas that are expressed in long-term culture. For Zeng (1996b), any cell population has two categories of individuals, one characterized with high protein productivity, and another with low protein productivity. The loss of producing capacity is attributed to mutation or loss of genetic materials, and is irreversible. The parameter "F_2" in Equation 70 represents the transformation rate from a producing to a non-producing population. The parameter "F_1" states a relationship between the specific production rates of these two populations, each one of them homogeneous in its productivity. Finally, an exponential function – $e^{F_2 \cdot \Delta t}$ – simulates the loss of producing capacity within a cell population.

If a process is operated in perfusion with high cell densities, Equation 70 might need an adjustment, as indicated in Equations 71 and 72. This can occur by taking the relation (S/X_V) for the main state variable, which

Table 8.8 Fitted parameters for models of Table 8.7

Eq.	References	α (mg/mg_{cel})	β ($mg/10^9$cel.h)	Other parameters
(65)	Pörtner *et al.*, 1996 Hiller *et al.*, 1991		0.0167 0.708	
(66)	Frame and Hu, 1991b	0.0266	0.00672 (mg/mg_{cel}.h)	
(67)	de Tremblay *et al.*, 1992		0.146	$\alpha_0 = 0.115$ mg/10^8cel $k_\mu = 8.33.10^{-4}$ h^{-1}
(68)	Linardos *et al.*, 1991			$\alpha_1 = 273$ g/10^6cel $\beta_1 = 0.0375$ g/10^6cel.h
(69)	Dalili *et al.*, 1990			$\beta_2 = 0.4$ mg/10^8cel.h $k_{GLN}^{MAb} = 0.1$ mM
(70)	Zeng, 1996b			$\alpha_1 = 0 \quad 51.6$ g/10^9cel $\beta_1 = 0.5 \quad 1.21$ mg/10^9cel.h $k_{GLN}^{MAb} = 4.42 \quad 9.01$ mM $k_{i,GLC}^{MAb} = 4.42 \quad 9.01$ mM GLC* = 0.1 mM $F_1 = 0.37 \quad 1.18$ $F_2 = 0 \quad 0.018$ h^{-1}
(71)	Zeng, 1996a			$\alpha_1 = 0.092$ mg/10^9cel $\beta_1 = 1.33$ mg/10^9cel.h $k_{GLN}^{MAb\#} = 0.0079$ mmol/10^9cel $k_{i,GLC}^{MAb\#} = 12.65$ mmol/10^9cel
(72)	Zeng, 1996a			$\delta = 4.16$ mg/10^9cel.h $k_{D_{Per}}^{MAb} = 0.115$ h^{-1} $k_{i,GLC}^{MAb} = 13.97$ mM

includes the influence of substrate and high cell density (Equation 71); or by considering the stimulation of productivity caused by the specific perfusion rate (D_{Per}).

Tables 8.9 and *8.10* show examples of kinetic expressions for byproduct synthesis and the parameter values fitted to them.

Frequently, the specific byproduct formation rate is presented as a function of specific substrate consumption rate and substrate-to-product yield (see Equation 12), but other structures can be assumed. The specific production rate can be limited by a precursor substrate and modeled by a Monod-type expression, as in Equation 73, or it may be inhibited by a substrate that is not, in principle, linked to its production, as in Equation

Table 8.9 Kinetic formulations for synthesis of byproducts (ammonia and lactate)

Formulations	References	Eq.
$\mu_{LAC} = \mu_{LAC,max} \cdot \frac{GLC}{k_{GLC}^{LAC} + GLC}$	Gaertner and Dhurjati, 1993	(73)
$\mu_{LAC} = Y_{LAC/X} \cdot \mu_X + m_{LAC} + \Delta\mu_{LAC,\,GLC}^{max} \cdot \frac{GLC \quad GLC^*}{GLC \quad GLC^* + k_{GLC}^{LAC}}$	Zeng, 1995	(74)
$\mu_{LAC} = Y_{LAC/X} \cdot \mu_X + m_{LAC} + \Delta\mu_{LAC,\,GLC}^{max} \cdot \frac{GLC}{GLC + k_{GLC}^{LAC\#} \cdot X_v} + \ldots$ $\ldots + \Delta\mu_{LAC,\,GLN}^{max} \cdot \frac{GLN}{GLN + k_{GLN}^{LAC\#} \cdot X_v}$	Zeng, 1996a	(75)
$\mu_{NH_3} = \frac{E_1 + E_2 \cdot GLC}{E_3 + GLC}$	Gaertner and Dhurjati, 1993	(76)
$\mu_{NH_3} = \frac{G_1 + G_2 \cdot LAC}{G_3 + LAC}$	Gaertner and Dhurjati, 1993	(77)
$\mu_{NH_3} = Y_{NH_3/X} \cdot \mu_X + m_{NH_3} + \Delta\mu_{NH_3,\,GLN}^{max} \cdot \frac{GLN3}{GLN3 + k_{GLN}^{NH_3}} + \ldots$ $\ldots + \Delta\mu_{NH_3,\,GLC}^{max} \cdot \frac{GLC}{GLC + k_{GLC}^{NH_3}}$	Zeng, 1995	(78)
$\mu_{NH_3} = Y_{NH_3/X} \cdot \mu_X + m_{NH_3} + \Delta\mu_{NH_3,\,GLN}^{max} \cdot \frac{GLN}{GLN + k_{GLN}^{NH_3\#} \cdot X_v}$	Zeng, 1996a	(79)

76 and 77. For example, glucose inhibits ammonia synthesis, an assumption that takes account of the complex regulation of dual-substrate metabolism (GLC and GLN) in animal cell systems (see Chapter 4).

In Equations 74 and 78 data fit to a model that considers the concept of excess substrate in culture, as set previously in Equation 36 (Zeng, 1995). However, this formulation needs modification in order to include high cell density conditions. In Equations 75 and 79, as seen before, the mass of nutrient available per cell (S/X_V) is the state variable to take this into account.

8.3.3 Parameter fitting in models

Unstructured models, as detailed in Sections 8.3.1 and 8.3.2, are formulated by a series of kinetic and differential non-linear equations that represent the dynamics of all the state variables during the process. Thus, to simulate a model that consists of parameters and state variables, it is necessary to attribute values to the parameters.

The initial parameters values can be estimated in different ways:

(i) from the stoichiometric relationship, utilizing experimental or theoretical data;

Table 8.10 Fitted parameters for models of Table 8.9

Eq.	Byproducts (bP)	$Y_{bP/X}\cdot\left(\frac{mmol_{sP}}{10^9 cel}\right)$	$m_{bP}\left(\frac{mmol_{sP}}{10^9 cel\cdot h}\right)$	$\Delta\mu^{max}_{bP,S}\left(\frac{mmol_{sP}}{10^9 cel\cdot h.}\right)$	k_S^{bP} (mM)	Other parameters
(73)	LAC				0.34	$\mu_{LAC,max} = 2.7$ (mmol/10^{10}cel.h)
(74)	LAC	10,42	0.050	0.355	0.43	
(75)	LAC	2553	888	1322 (S = GLN) 3663 (S = GLC)		$k_{GLN}^{LAC\#} = 0.013$ (mmol/10^9cel) $k_{GLC}^{LAC\#} = 17.54$ (mmol/10^9cel)
(76)	NH_3					$E_1 = 2.0$ (mmol^2/10^{10}cel.L.h) $E_2 = 0.35$ (mmol/10^{10}cel.h) $E_3 = 3.3$ mM
(77)	NH_3					$G_1 = 6.2$ (mmol^2/10^{10}cel.L.h) $G_2 = 0.22$ (mmol/10^{10}cel.h) $G_3 = 14$ mM
(78)	NH_3	0.21	0.00068	0.032 (p/S = GLN) 0.0041 (p/S = GLC)	0.56 (p/GLN) 0.96 (p/GLC)	.
(79)	NH_3	0.73		0.025 (S = GLN)		$k_{GLN}^{NH_3\#} = 0.071$ (mmol/10^9cel)

(ii) by linearization and, sometimes, simplification of kinetic equations and by fitting the experimental data by linear regression;
(iii) by utilizing parameter values available in the literature.

Item (i) above is largely employed to obtain substrate-to-cell and substrate-to-product yield factors. When the substrate consumption is diverted to several different products, an estimate of the substrate-to-product yield factors can be based on the stoichiometric relationships obtained from the biochemical reactions.

Item (ii) is largely employed in obtaining kinetic parameters such as the substrate limitation and inhibition constants for cellular growth and death (k_S and $k_{i,S}$, respectively) and substrate inhibition constants for production ($k_{i,S}^P$); maximum specific growth and death rates ($\mu_{X,max}$ and $k_{d,max}$), as well as the global yield factors ($Y_{X/S}$ and $Y_{P/S}$), that may be associated or not to the cellular growth.

Simplification of the equations involves separating the parameters to be estimated.

Besides the simplification, a subset of data must be selected, from the available data set, that is more adequate for the determination and fitting of the parameters.

Andrew's kinetic model (Equation 21) (Andrews, 1968), for example, relates the specific growth rate (μ_X) with the substrate concentration S, applying three parameters ($\mu_{X,max}$, k_S and $k_{i,S}$).

The initial parameter estimations of $\mu_{X,max}$ and k_S can be based on a simplification of Equation 21, by disregarding the inhibition term for the substrate. Thus, Equation 21 becomes Equation 80.

$$\mu_X = \mu_{X,max} \frac{S}{k_S + S} \tag{80}$$

Re-arranging this equation results in its linearization.

$$\frac{1}{\mu_X} = \frac{1}{\mu_{X,max}} + \frac{k_S}{\mu_{X,max}} \cdot \frac{1}{S} \tag{81}$$

Using data extracted from the test conducted with non-inhibiting substrate concentrations, the construction of the graph $1/\mu_X = a + b \cdot (1/S)$ allows a fit of values for "a" and "b" and, from these, an estimate of $\mu_{X,max}$ and k_S (Equation 81).

An initial estimate for parameter $k_{i,S}$ can be obtained by the simplification of Equation 21, disregarding the limitation for the substrate term, S, forming Equation 82:

$$\mu_X = \mu_{X,max} \frac{S}{S + \dfrac{S^2}{k_{i,S}}} \tag{82}$$

Re-arranging this equation results in its linearization:

$$\frac{1}{\mu_X} = \frac{1}{\mu_{X,max}} + \left(\frac{1}{\mu_{X,max.} k_{i,s}} \right) \cdot S \tag{83}$$

Using data extracted from the test conducted with inhibiting substrate concentrations, the construction of the graph $1/\mu_X = a + b \cdot S$ allows a fit of values for "a" and "b", and, from these, an estimate of $\mu_{X,max}$ and $k_{i,S}$ (Equation 83). Others ways of estimating parameters can be found in Bonomi and Schmidell (2001).

Once the initial values of the parameters are estimated, the model becomes dependent only on the state variable. From now on, forecasts can be made with the model and the results compared to the experimental data for different culture conditions (model simulation). Thus, the quality of

the model is evaluated and, if necessary, one should try new values for the parameters to improve the forecasts so that they resemble more and more the experimental values. This approach is named model fitting and intends to find the optimal parameters that minimize the difference between the experimental data and that generated by the model. The final result of this fitting is the final model parameter estimation, also called parameter optimization.

The estimation of model parameters can be reduced to the solution of an optimization problem. This means that once the objective is defined, a class of mathematical functions that best match the specified objectives must be found.

The optimization can be carried out by several methods of linear and nonlinear regression. The mathematical methods must be chosen with criteria to fit the calculation of the applied objective functions. The most widely applied methods of nonlinear regression can be separated into two categories: methods with or without using partial derivatives of the objective function to the model parameters. The most widely employed nonderivative methods are zero order, such as the methods of direct search and the Simplex (Himmelblau, 1972). The most widely used derivative methods are first order, such as the method of indirect search, Gauss-Seidel or Newton, gradient method, and the Marquardt method.

The objective functions employed in the parameter optimization can assume different forms (Equations 84 to 89):

$$OF = \sum_i (\tilde{y}_i - y_i)^2 \tag{84}$$

where:
OF = objective function;
y_i= experimental value of variable *i*.

Or

$$OF = \sum_i \left(\frac{\tilde{y}_i}{(y_i)_m} - \frac{y_i}{(y_i)_m} \right)^2 \tag{85}$$

where:
$\tilde{y}_i$= calculated value of variable *i*;
$(y_i)_m$= maximum experimental value of variable *i*.

Or

$$OF = \sum_i \left(\frac{\tilde{y}_i - y_i}{\tilde{y}_i} \right)^2 \tag{86}$$

Or

$$OF = \sum_i \left[\frac{\tilde{y}_i - y_i}{\left(\frac{\tilde{y}_i - y_i}{2} \right)} \right]^2 \tag{87}$$

Or

$$OF = \sum|r - 1| + \sum|a - 1| \tag{88}$$

where:

r = correlation coefficient of the linear regression (experimental vs. calculated variables);

a = angular coefficient of the linear regression (experimental vs. calculated variables).

$$OF = |r - 1| + |a - 1| \tag{89}$$

Whatever the form of calculation of the objective function or the method employed in parameter optimization, the numerical solution of the set of differential equations can be obtained by applying algorithms of initial values. Examples of these algorithms are the implicit integration methods (Euler, Crank-Nicolson, Adams-Moulton), or prediction-correction methods (Runge-Kutta, Michelsen, Gear) or bounded value problem algorithms, such as finite difference methods, Jacobi's polynomial or orthogonal collocation methods (Rice and Do, 1995; Constantinides and Mostoufi, 1999).

In the Appendix of this book, there is an example of parameter fitting proposed for the case of mathematical modeling of mAb production, adapted from Lee (2003).

8.3.4 Model validation

The next step to model fitting is its validation. Statistical analysis is employed for the verification of the experimental error, determination of the best model, to identify any systematic deviation between the fitted model and the experimental data, as well as to verify whether the model is statistically consistent, that is, whether it shows an error in the real data consistent with the experimental error.

The most widely employed statistical test for validation of a mathematical model is the F test.

For the application of this test, the experimental error must be known. One way of determining the experimental error is to carry out a set of tests, conducted in identical conditions. The relationship between the error from model fitting and the experimental error estimate is given by Equation 90:

$$F_c = \frac{s_c^2}{s_e^2} \tag{90}$$

where:

s_c^2 = model error for variance estimate;

s_e^2 = experimental error variance estimate.

The form of calculation of these variances is given by Equations 91 and 92.

$$s_c^2 = \frac{\sum_{i=1}^{v}\sum_{j=1}^{n}(\tilde{y}_{ij} - y_{ij})^2}{(nv)_c - p} \quad (91)$$

$$s_e^2 = \frac{\sum_{i=1}^{v}\sum_{j=1}^{n}(y_{ij} - \overline{y}_i)^2}{(nv)_e - v} \quad (92)$$

where:

n = number of points for each variable;
v = number of state variables defined by the model;
$(nv)_c$ = number of fitted points (all tests and variables);
$(nv)_e$ = number of experimental points (all repeated tests and state variables);
p = number of parameters from the model;
$\tilde{y}_{ij}$= variable values calculated by the model;
y_{ij}= experimental variable values;
$\overline{y}_i$= average variable values for repeated tests.

For model validation, $F_c < 1$ or $s_c^2 < s_e^2$.

For more than one model, the relationship of the model to experimental data can be determined by the χ^2 Bartlett statistical test (Fromment and Bischoff, 1990).

8.4 Structured and non-segregated models

The structured and non-segregated models, as mentioned before, consider the cell population as homogeneous, but the intracellular structures are separated, in a way that the cell is considered complex and treated as a heterogeneous structure. Thus, it is possible to characterize the intrinsic cell behavior and to forecast its interaction. As indicated in Chapter 5, media for animal cells culture are complex, with components that include sugar, amino acids, salts, and growth factors. Glucose and glutamine added to media as carbon, nitrogen, and energy sources can affect cellular growth and byproducts (such as lactate and ammonia) can accumulate in the culture medium causing inhibition.

The difficulty of understanding all these interactive phenomena from the cell and environment, which are in permanent transition, demands a detail of the cellular structure – the characteristic of the structured models. Depending on the quantity of detail and physiological structures considered, these structured models have varying ability for forecasting and become highly valuable tools for understanding the cell physiology.

One of the pioneers of structured models in animal cell culture used a single-cell model (Batt and Kompala, 1989). Based on hybridoma metabolism (*Figure 8.6*), the model was based on the formulation of four compartments: amino acids (including the TCA precursors), the nucleotides (including DNA and RNA), the proteins, and lipids. The excreted byproducts (lactate and ammonia) and the excreted product (mAb) were also considered. However, although flexible for simulation of different

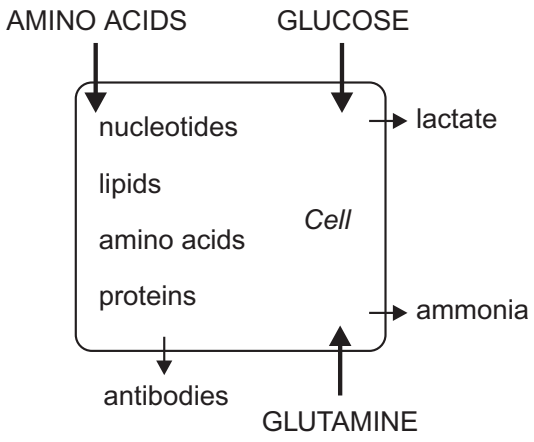


Figure 8.6

Hybridoma metabolism scheme (based on Batt and Kompala, 1989).

operating strategies, the model lacked detail concerning inhibition by secreted byproducts.

Different structured models formulated for hybridoma growth have been shown (Linardos *et al*., 1992; Cazzador and Mariani, 1993; Martens *et al*., 1995; Sanderson *et al*., 1999), as well as production of mAbs (Suzuki and Ollis, 1990).

Besides being more robust than the unstructured models, these structured models describe the cellular response under a narrow band of conditions. To forecast the cellular response to operation strategies of various bioreactors, the model can be extended by an increasing number of structures, to accumulate metabolic information (Wu *et al*., 1992) or associate the metabolic fluxes to the structured model (Follstad *et al*., 1999). The methodologies for flux balances, which employ a series of reactions from glucose metabolism (*Figure 8.7*), and calculate all the metabolic reactions rates during the stationary state, allow a better understanding of the cellular behavior and the prediction of other more efficient operating conditions.

Evidently, a higher number of structures implies that a higher number of equations and parameters must be employed to describe the process kinetics, increasing the difficulties in formulation and quantification of the intracellular components. Even so, the capacity for identifying these intrinsic cellular phenomena and their incorporation into the models increases the ability for prediction and forecasting. This forms an important part of the study and development of the kinetic models.

8.5 Unstructured and segregated models

The representation of a property variation (mass, age, volume, etc.) within a cellular population is made using population balance models (PBM). These models allow the calculation of the variation of the rate of a population characteristic considering the rates of increase and disappearance of the specific characteristic within the cell population. Thus, they allow a calculation of one special feature in the population. Mathematical models that were originally unable to consider the diversity of character-

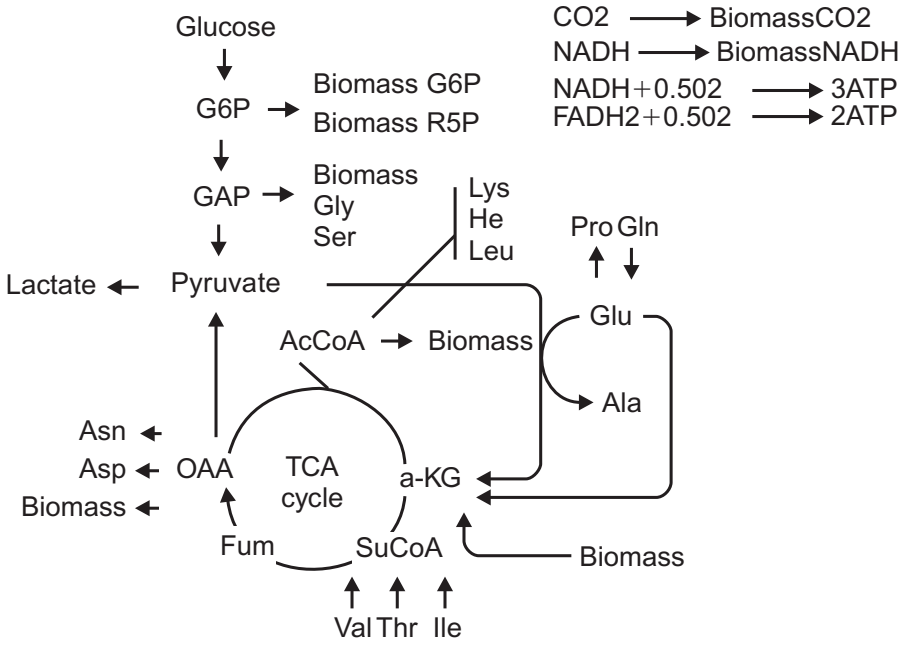


Figure 8.7

Hybridoma metabolism scheme (based on Follstad et al., 1999).

istics can now take this into account. The inclusion of cellular diversity makes the model more complex and it is necessary to use non-trivial mathematics to solve the equations, including the integration of partial differential equations. Sidoli *et al*. (2004) have written a review of PBMs for animal cells, indicating the major segregated models and their characteristics. These models are classified into single- or multi-variables, depending on the number of properties considered in the model, as well as single- or multi-staged models, depending on the number of phases considered in the cellular life cycle. If the mass is the property variation to be considered, then the model is called **mass structured**, and if the cellular age is the property to be considered then the model is called **aged structured**.

As an example, the single-variable population balance model, single-staged, mass structured is indicated by Equation 93.

$$\overbrace{\frac{\partial N(m,t)}{\partial t}}^{\text{Accumulation}} = -\overbrace{\frac{\partial\left[r(m,S)N(m,t)\right]}{\partial m}}^{\text{Single cell growth}} \overbrace{-\Gamma(m,S)N(m,t)}^{\text{Celullar division rate}} + \overbrace{2\int_{\tau}^{\infty}\Gamma(m',S)p(m,m',S)N(m',t)dm}^{\text{Birth rate}} \tag{93}$$

This equation states that accumulation of cells with a mass ***m*** is the consequence of three rates that determine the dynamics of the mass variation in the population: the rate of reduction of the number of elements with mass ***m*** due to the mass increase that results from cell

growth; the rate of reduction of the number of cells due cellular division, and the rate of generation of new cells within this mass ***m***.

In this equation **N**(m, t) is the distribution indicating the number of cells with mass *m,* a function that depends on the mass of the cell, because there is a mass distribution in the population, and on time, since there are factors that continuously modify this distribution over time. Thus, **N**(m, t) is a distribution function of a property in the population, in this in case the cellular mass, over time.

The growth of cells of mass ***m*** is indicated by function **r**(m, S), in Equation 93. It is a cell growth rate dependent on the cell mass and also on the limiting substrate concentration. If a cell increases its mass, as a consequence it leaves the class of the cells of mass ***m***.

The cellular division also causes the reduction of the number of cell with mass ***m*** and this reduction is determined by a division rate, **Γ**(m, S) in Equation 93. The product **Γ.N** indicates the division rate of all the cells with a mass ***m***. Cell division gives rise to two daughter cells from the mother cells of mass **m′**. The information on how the parent cell partitions its mass off is contained in the partition function **p**(m, m′, S), that expresses the probability of a parent cell of mass ***m′*** giving rise to a daughter cell of mass ***m***. In this way, **2.p.Γ.N** indicates the rate of appearance of new cells with mass ***m***, originating from parent cells of mass **m′**. The parent cells have a continuous distribution of mass that goes from a minimum mass, m_{min}, to an unlimited value (theoretically to an infinite mass). Consequently, the total rate of cell generation with mass ***m*** is calculated by the integration of cell generation rates for all intervals of variation of the mass in the population. The division rate for the whole distribution is the third term of Equation 93.

To solve Equation 93 it is necessary to know the characteristic functions of the cellular physiology, that is, the growth rate **r**(m, S), the cellular division rate **Γ**(m, S), and the cell mass partition function **p**(m, m′, S). Since these functions are substrate concentration- dependent, the substrate consumption rate must also be defined. These substrate concentration variations are calculated using the yield coefficient $Y_{X/S}$, that establishes a relationship between the growth rates and the substrate consumption rate. The consumption rate is indicated by Equation 94.

$$\frac{dS}{dt} = \frac{1}{Yx/s} \times \int_{m_{min}}^{\infty} r(m, S) \times N(m, t)\ dm \qquad (94)$$

As pointed out before when discussing structured models, physiological functions like the single cell growth rate **r**(m, S), cellular division rate **Γ**(m, S), and cell mass partition function **p**(m, m′, S) are difficult to measure and this adds to the complexity of using the model. The mathematical complexity is also a limitation for optimization and control purposes that demand online measurements and calculations.

The difficulties of characterizing the cell physiological properties can explain the difficulties in applying these models and why they have not been widely used in the solution of biochemical engineering problems. On the other hand, the advances in monitoring, especially in flow cytometry, and in computer science development, that increase the capacity for model

prediction, have provided new stimulating factors that have made these models an option for some researchers.

References

Aiba S, Shoda M (1969), Reassessment of product inhibition in alcohol fermentation, J. Ferment. Technol. 47:790 794.

Aiba S, Shoda M, Nagatami M (1968), Kinetics of product inhibition in alcohol fermentation, Biotechnol. Bioeng. 10:845 864.

Andrews JF (1968), A mathematical model for the continuous culture of microrgan isms utilizing inhibitory substrate, Biotechnol. Bioeng. 10:707 723.

Bailey JE, Ollis DF (1986), Biochemical engineering fundamentals, 2nd Ed., McGraw Hill, New York.

Batt BC, Kompala DS (1989), A structured kinetic modeling framework for the dynamics of hibrodoma growth and monoclonal antibody production in contin uous suspension cultures, Biotechnol. Bioeng. 34:515 531

Bonomi A, Schmidell W (2001), Modelagem matemática e simulação de processos fermentativos, In: Schmidell W, Lima UA, Aquarone E, Borzani W (Eds), Biotecnologia Industrial, vol. 2, Engenharia Bioquímica, Edgard Blücher, Brazil, pp. 123 178.

Bree MA, Dhurjati P, Geoghegan R, Robnett B (1988), Kinetic modeling of hybridoma cell growth and immunoglobulin production in a large scale suspensions culture, Biotechnol. Bioeng. 32:1067 1072.

Cazzador L, Mariani J (1993), Growth and production modeling in hybridoma continuous cultures, Biotechnol. Bioeng. 42:1322 1330.

Constantinides A, Mostoufi N (1999), Numerical Methods for Chemical Engineers with MATLAB Applications, Prentice Hall, New Jersey.

Contois DE (1959), Kinetics of bacterial growth: relationship between population density and specific growth rate of continuous cultures, J. Gen. Microbiol. 21: 40 50.

Dalili M, Sayles GD, Ollis DF (1990), Glutamine limited batch hybridoma growth and antibody production: experiment and model, Biotechnol. Bioeng. 36:74 82.

de Tremblay M, Perrier M, Chavarie C, Archambault J (1992), Optimization of fed batch culture of hybridoma cells using dynamic programming: single and multi feed cases, Bioprocess Eng. 7:229 234.

Dunn IJ, Heinzle E, Ingham J, Prenosil, JE (1992), Biological Reaction Engineering, VCH.

Engasser JM, Goergen JL, Marc A (1998), Modeling, In: Doyle A, Griffiths JB (Eds), Cell and Tissue Culture: Laboratory Procedures in Biotechnology, John Wiley & Sons, Guildford, UK, pp.160 178.

Follstad BD, Balcarcel RR, Stephanopoulos G, Wang DIC (1999), Metabolic flux analysis of hybridoma continuous culture steady state multiplicity, Biotechnol. Bioeng. 63:675 683.

Frame KK, Hu WS (1991a), Kinetic study of hybridoma cell growth in continuous culture. I. A model for non producing cell, Biotechnol. Bioeng. 37:55 64.

Frame KK, Hu WS (1991b), Kinetic study of hybridoma cell growth in continuous culture. II. Behaviour of producers and comparison to non producers, Biotechnol. Bioeng. 38:1020 1028.

Freshney RI (1994) Culture of Animal Cells. A Manual of Basic Technique, 3rd Ed., Wiley Liss, New York.

Fromment GF, Bischoff KB (1990), Chemical reactor analysis and design, John Wiley & Sons, Singapore.

Gaertner JG, Dhurjati P (1993), Fractional factorial study of hybridoma behavior. 2. Kinetics of nutrient uptake and waste production, Biotechnol. Prog. 9:309 316.

Ghose TK, Tyagi RD (1979), Rapid ethanol fermentation of cellulose hydrolysate. II. Product and substrate inhibition and optimization of fermentor design, Biotech nol. Bioeng. 21:1401 1420.
Glacken MW, Huang C, Sinskey AJ (1989), Mathematical descriptions of hybridoma culture kinetics. III Simulation of fed batch bioreactors, J. Biotechnol. 10:39 66.
Harigae M, Matsumura M, Kataoka H (1994), Kinetic study on HBs MAb production in continuous cultivation, J. Biotechnol. 34:227 235.
Hiller GW, Aeschlimann AD, Clark DS, Blanch HW (1991), A kinetic analysis of hybridoma growth and metabolism in continuous suspension culture on serum free medium, Biotechnol. Bioeng. 38:733 741.
Himmelblau DM (1972), Applied Nonlinear Programming, McGraw Hill, New York.
Hiss H (2001), Cinética de processos fermentativos, In: Schmidell W, Lima UA, Aquarone E, Borzani W (Eds), Biotecnologia Industrial, vol. 2, Engenharia Bioquímica, Edgard Blücher, Brazil, pp. 93 122.
Kurokawa H, Park YS, Iijima S, Kobayashi T (1994), Growth characteristics in fed batch culture of hybridoma cells with control of glucose and glutamine concentra tions, Biotechnol. Bioeng. 44:95 103.
Lancaster P, Salkauskas K (1986), Curve and Surface Fitting, Academic Press, London.
Le Duy A, Zajic JE (1973), A geometrical approach for differentiation of an experi mental function at a point: applied to growth and product formation, Biotechnol. Bioeng. 15:805 810.
Lee CY (2003), Estratégias para otimização da produção de anticorpo monoclonal anti TNP (trinitrofenil) em sistema de biorreator agitado: fortificação do meio e processo descontínuo alimentado. Dissertação de Mestrado, Programa de Mestra do em Biotecnologia Interunidades USP I.Butantan IPT.
Lee YK, Yap PK, Teoh AP (1995), Correlation between steady state cell concentration and cell death of hybridoma cultures in chemostat, Biotechnol. Bioeng. 45:18 26.
Linardos TI, Kalogerakis N, Behie LA, Lamontagne LR (1991), The effect of specific growth rate and death rate on monoclonal antibody production in hybridoma chemostat cultures, Can. J. Chem. Eng. 69:429 438.
Linardos TI, Kalogerakis N, Behie LA (1992), Cell cycle model for growth rate and death rate in continuous suspension hybridoma cultures, Biotechnol Bioeng. 40:359 368.
Luedeking R, Piret EL (1959), A kinetic study of the lactic acid fermentation. Batch process at controlled pH, J. Biochem. Microbiol. Technol. Eng. 1:393 412.
Martens DE, Sipkema EM, de Gooijer CD, Beuvery EC, Tramper J (1995), A combined cell cycle and metabolic model for the growth of hybridoma cells in steady state continuous culture, Biotechnol. Bioeng. 48:49 65.
Megee RD, Drake JF, Fredrickson AG, Tsuchiya HM (1972), Studies in intermicrobial symbiosis of *Saccharomyces cerevisiae* and *Lactobacillus casei*, Can. J. Microbiol. 18:1733 1742.
Miller WM, Reddy A (1998), Quantitative analysis of cell growth, metabolism, and product formation, In: Doyle A, Griffiths JB (Eds), Cell and Tissue Culture: Laboratory Procedures in Biotechnology, John Wiley & Sons, Guildford, UK, pp. 133 159.
Miller WM, Blanch HW, Wilke CR (1988), A kinetic analysis of hybridoma growth and metabolism in batch and continuous suspension culture: effect of nutrient concentration, dilution rate, and pH. Biotechnol. Bioeng. 32:947 965.
Monod J (1942), Recherches sur la Croissance de Culture Bacterienne, Hermann & Cie, Paris.
Monod J (1949), The growth of bacterial cultures, Annu. Rev. Microbiol. 3:371 394.
Moser H (1958), The dynamics of bacterial population maintained in the chemostat. Carnegie Institution of Washington, Washington.
Nielsen J, Nikolajsen K (1988), Review and discussion of literature on mathematical

models on microbial systems, Status Report 2: Structure models for microbial systems, Department of Biotechnology, Technical University of Denmark, Den mark,
Pirt SJ (1966), The maintenance requirement of bacteria in growing cultures, Proc. R. Soc. Lond. B 163:224 231.
Pörtner R, Schäfer T (1996), Modeling hybridoma cells growth and metabolism a comparison of selected models and data, J. Biotechnol. 49:119 135.
Pörtner R, Schilling A, Lüdemann I, Märkl H (1996), High density fed batch cultures for hybridoma cells performed with the aid of a kinetic model, Bioprocess. Eng. 15:117 124.
Rice RG, Do DD (1995), Applied Mathematics and Modeling for Chemical Engineers, John Wiley & Sons, New York.
Sanderson CS, Jang JD, Barford JP, Barton GW (1999), A structured, dynamic model for animal cell culture systems: application to murine hybridoma, Biochem. Eng. J. 3:213 218.
Sidoli FR, Mantalaris A, Asprey SP (2004), Modeling of mammalian cells and cell culture processes, Cytotechnology 44:27 46.
Sinclair CG, Kristiansen B (1987), Fermentation kinetics and modeling, Open Uni versity Press, Taylor & Francis, UK.
Suzuki E, Ollis DF (1990), Enhanced antibody production at slowed growth rates: experimental demonstration and a simple structured model, Biotechnol. Prog. 6:231 236.
Tsai SP, Lee YH (1990), A model for energy sufficient culture growth, Biotechnol. Bioeng. 35:138 145.
Tsuchiya HM, Fredrickson AG, Aris R (1966), Dynamics of microbial cell population, Adv. Chem. Eng., 6:125 206.
Tziampazis E, Sambanis A (1994), Modeling of cell culture processes, Cytotechnology, 14:191 204.
Wu P, Ray NG, Shuler ML (1992), A single cell model for CHO cells, Ann. N Y Acad. Sci., 665:152 187.
Wu YC, Hao OJ, Ou KC, Schoelze RJ (1988), Treatment of leachate from solid waste landfill site using a two stage anaerobic filter, Biotechnol. Bioeng. 31:257 266.
Zeng AP (1995), A kinetic model for product formation of microbial and mammalian cells, Biotechnol. Bioeng, 46:314 324.
Zeng AP (1996a), Quantitative assessment of cell density effect on the metabolism and antibody production rate of hybridoma cells at high cell density, J. Biotechnol. 45:243 251.
Zeng AP (1996b), Mathematical modeling and analysis of monoclonal antibody production by hybridoma cells, Biotechnol. Bioeng. 50:238 247.
Zeng AP, Deckwer WD (1995a), A kinetic model for substrate and energy consump tion of microbial growth under substrate sufficient conditions, Biotechnol. Prog. 11:71 79.
Zeng AP, Deckwer WD (1995b), Mathematical modeling and analysis of glucose and glutamine utilization and regulation in cultures of continuous mammalian cells, Biotechnol. Bioeng. 47:334 346.
Zeng AP, Deckwer WD, Hu WS (1998), Determinants and rate laws of growth and death of hybridoma cells in continuous culture, Biotechnol. Bioeng. 57:642 654.

Bioreactors for animal cells

9

Ernesto Chico Véliz, Gryssell Rodríguez, and Alvio Figueredo Cardero

9.1 Introduction

Around the end of the 1970s it became apparent that cells could be cultivated *in vitro* on a large scale for the production of a wide range of human health products. Since then, studies aimed at defining the relevant environmental conditions needed for efficient cell proliferation *in vitro* have been increasingly carried out. This has motivated work on the development of chemically defined media and on the determination of optimal physicochemical conditions (pH, temperature, dissolved oxygen, etc.) for culturing cells for large-scale operations. As a practical consequence of these studies, several types of equipment to control environmental culture conditions have been developed, giving rise to the bioreactor systems currently used in the biopharmaceutical industry.

To ensure adequate cell proliferation and product biosynthesis, bioreactors have to meet the following requirements:

(i) control the acid-base equilibrium (pH) of the culture medium;
(ii) control temperature;
(iii) provide gas exchange to appropriately supply oxygen to the cells and remove excess carbon dioxide;
(iv) allow the supply of nutrients through the use of adequate formulations of culture media;
(v) supply enough surface for adhesion of cells, in the case of anchorage-dependent cells;
(vi) maintain aseptic conditions, avoiding contamination by microorganisms, viruses, or other cells.

The basic challenge regarding the operation of bioreactors is to guarantee a homogeneous physicochemical environment for the whole cell population, preventing the occurrence of large gradients and regions with inadequate conditions, which could cause the undesired death of part of the cell population. The maintenance of adequate environmental conditions requires the use of sensors and control systems, as discussed in detail in Chapter 10.

9.2 Inoculum propagation and small-scale culture systems

To ensure consistency and reproducibility, especially in industrial production processes, it is important always to use raw materials with identical

properties. Therefore, it is necessary, in each production lot, to employ the same cell line and guarantee that cell characteristics do not change between lots. Also, in research, it is important to use defined cell lines and to ensure that they do not change by mutations over time that could alter their properties.

To guarantee the availability of cells and the maintenance of their characteristics, a sufficiently large quantity of cells is kept in master and working cell banks, as discussed in Chapters 13 and 14. The number of cryotubes containing cells in a working cell bank is calculated to ensure that, for the whole expected lifespan of a product, each production lot will be obtained starting with cells from identical cryotubes. The most widely employed method for the conservation of cell banks is cryoconservation in liquid nitrogen at –196°C, which avoids mutations that could potentially alter cell characteristics.

Each production lot starts by thawing cells from a cryotube, which usually contains 10 to 20 million cells suspended in a volume of 1–10 mL. Thawing cells is a stage of the process that should be carried out with great care, since it can generate a high level of cell death if performed inadequately.

After eliminating the cryoprotector (generally DMSO – dimethyl sulfoxide), cells are suspended in fresh medium at a concentration between 0.1 and 0.4 million cells per-mL, depending on the cell line and the culture medium, to be used subsequently for incubation and propagation. This range of inoculum concentration is used as a standard for the inoculation of animal cell cultures, since lower concentrations result in long lag phases and in a delay in cell growth, which is detrimental to process time and productivity.

In general, the subsequent stages occur in small bioreactors, known simply as flasks or bottles, since they have no monitoring and control system and are usually disposable. Apart from flasks and bottles, multiwell plates can be used to culture cells, but their use is restricted mainly to cloning and selection of cells.

In the first step after thawing, cells are generally propagated in stationary flasks with approximately 10 ml working volume, with a monolayer of cells being formed on the lower surface of the flask after 2–3 days. The cells are later transferred to larger flasks, maintaining the cell concentration range mentioned above. Alternatively, cells able to grow in suspension can be thawed directly from the cryotube to small stirred flasks, such as magnetically stirred flasks known as spinners.

In stationary flasks, oxygen transfer to the liquid medium is limited by the small gas–liquid interface area and by the lack of agitation. For this reason, other types of culture systems need to be used when working with more than 100 million cells. The type of culture system to be used depends on the cell line characteristics, mainly if it is adherent or if it is able to grow in suspension (*Figure 9.1*). For cells growing in suspension, spinner flasks are largely used, although shaker flasks may also be employed. Anchorage-dependent cells, on the other hand, are frequently cultured in roller bottles. For the transfer of adherent cells from one flask to another, cells can be released mechanically or enzymatically, e.g. using trypsin.

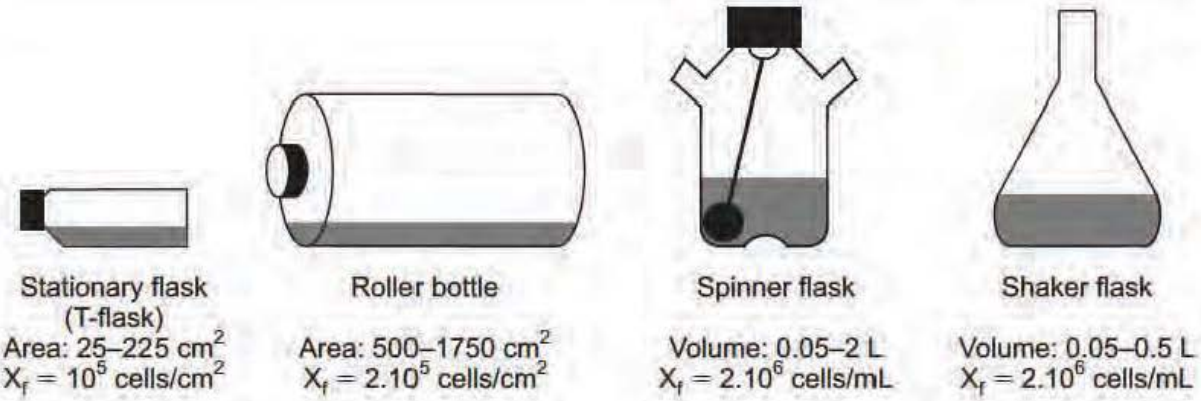


Figure 9.1

Low volume bioreactors. Although it is mentioned in literature that stirred flasks can be used up to 20 L, they are mostly used for lower volumes (up to 2 L). Xf: typical final cell concentration.

All the procedures carried out on a small scale are labor-intensive and require skilled operators. Manipulation of the cultures is carried out in laminar-flow cabinets, to maintain asepsis. During this stage of cell propagation/inoculum development, activities are carried out by direct operator manipulation, so that the process is considered to be "open". Thus, as a general rule, these activities occur in areas separated from those where the intermediate and production bioreactors are installed.

Figures 9.2 and *9.3* show schemes that illustrate inoculum development from the cryotubes to production scale for suspension and adherent cells, respectively. In these hypothetical process schemes, the expression "production bioreactor" is used arbitrarily for any of the types of bioreactor presented in the next section of this chapter. In general, different flasks and several intermediate bioreactors are used for cell propagation to reach the quantity of cells necessary to inoculate the production bioreactor. The number of propagation steps is a function of the final scale of the production bioreactor.

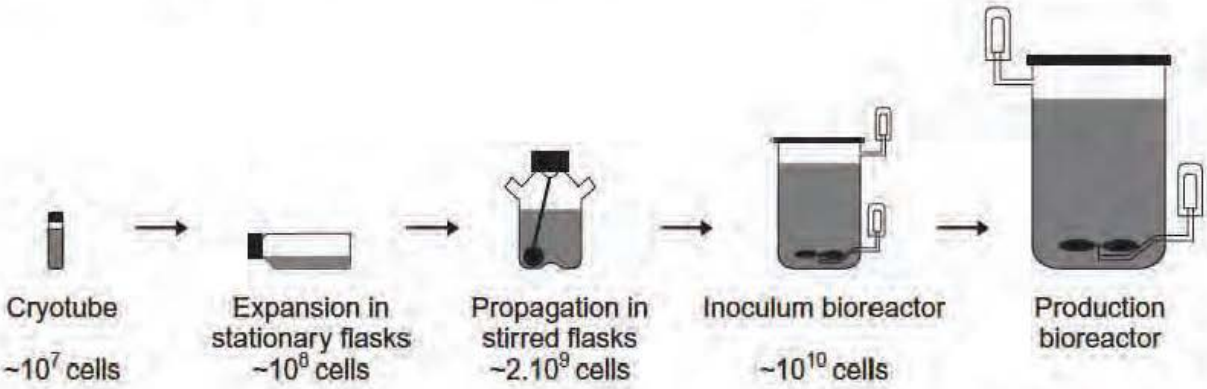


Figure 9.2

Hypothetical example of the propagation of suspension cells, using stirred tank bioreactors in the intermediate and final stages. The amount of cells usually obtained at the end of each step is also indicated.

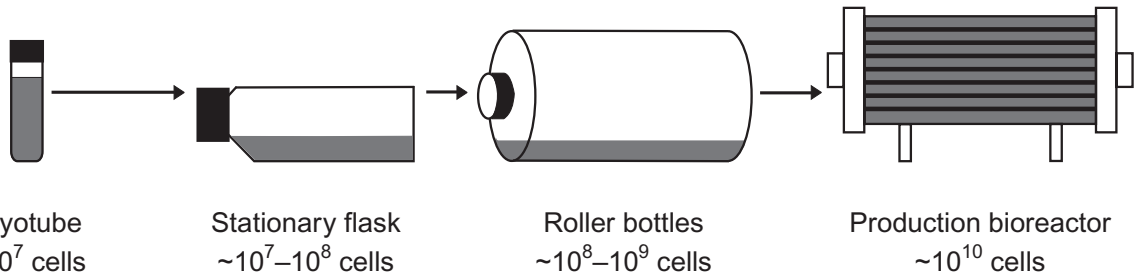


Figure 9.3

Hypothetical example of the propagation of adherent cells, using hollow fiber modules as the production bioreactor in the final stage. The amount of cells usually obtained at the end of each step is also indicated.

9.3 Types of bioreactors

In the last few decades, a wide variety of bioreactors have been developed, with some of them being an attempt to mimic animal organs, and others being an adaptation of the principles of submerged microbial fermentation to the culture of animal cells.

The bioreactors used for animal cell cultivation can be classified according to different criteria. One of the most useful refers to the homogeneity of the system. All culture systems are multiphase systems, since at least two phases are involved: a solid one (cells) and a liquid one (culture medium). Eventually, a third phase (gas) may be present due to bubble aeration. Bioreactors in which cells are uniformly suspended in the liquid phase are designated homogeneous bioreactors. On the other hand, those bioreactors in which cells are not homogeneously distributed in the entire volume are called heterogeneous bioreactors. *Table 9.1* lists different types of bioreactors classified according to the homogeneity of the system.

Given the potential risk of contamination of a biopharmaceutical product with residues of parts of a bioreactor in contact with the culture, the materials used in the construction of bioreactors must respect the rules dictated by the regulatory agencies of different countries. The manufacturing process of the components of a bioreactor must be well documented and the materials must be certified.

In the case of stainless steel, the surfaces that come in contact with the product must be made of pharmaceutical-grade stainless steel (AISI 316L). The average surface rugosity (Ra), defined as the average distance of the base of a surface up to the rugosity profile (or the average height of bumps on a surface), must be lower than 0.8 μm. Thus, different treatments are employed for polishing the surfaces, with the final aim of facilitating equipment cleaning and maintaining process asepsis. Among the treatments commonly employed, electropolishing is recommended for final surface manipulation. This treatment does not alter macrorugosity of a surface, but it smooths its profile and adds a dense inert layer of chromium oxide, which protects the metal from corrosion. Furthermore, electropolishing eliminates hydrogen from the polished surface, decreasing the

ability of microorganisms to grow on it. *Figure 9.4* (see color section) shows a polished surface inside an animal cell stirred-tank bioreactor.

Seals, o-rings, and valve membranes must be manufactured out of polymers that are resistant to thermal sterilization and to the bases and acids used in the biopharmaceutical industry. When in contact with the product, the elastomers employed must additionally present the following safety characteristics: the rates of release of polymer components must be low and remain below well-established threshold levels; the components released must be innocuous to human health, according to rules previously established.

Another material that is frequently employed is glass. It is used in small-scale vessels and also in sight glasses of large-scale bioreactors and tanks. A detailed review of the materials used in the manufacturing of bioreactors, as well as the surface treatments used, can be found in the literature (Krahe, 2003).

9.3.1 Homogeneous bioreactors

Stirred-tank, air-lift, and wave bioreactors are the most important types of homogeneous bioreactors used in animal cell cultivation, as previously outlined in *Table 9.1*. One of the most important advantages of using homogeneous bioreactors is the possibility of monitoring and controlling the physicochemical environment to which the cells are submitted in a much more reliable way than in heterogeneous bioreactors. Thus, it is possible to indirectly control the physiological state of cells.

Stirred-tank bioreactors

Stirred-tank bioreactors are widely used in the modern biotechnological industry. Most products produced from animal cells on a large scale worldwide are manufactured in this type of bioreactor. In general, these reactors are very similar to fermenters used in industrial submerged culture of microorganisms, and are simple to design, having the shape of a tank and impellers to promote mixture of the contents.

Stirred-tank bioreactors can be operated according to any of the operation modes that will be discussed later in this chapter. *Figure 9.5A* (see color section) shows the typical design of a stirred-tank bioreactor, fitted with a continuous supply of gases for oxygenation and pH control and with a gas exhaust outlet in the upper part of the equipment. In most cases, oxygenation occurs by bubbling gases below the impeller, as will be

Table 9.1 Classification of bioreactors according to system homogeneity

Homogeneous bioreactors	Heterogeneous bioreactors
Stirred tank bioreactors	Microcarrier based systems
Air lift bioreactors	Packed bed bioreactors
Wave bioreactors	Fluidized bed bioreactors
	Hollow fiber bioreactors
	Bioreactors providing surfaces for attached cell growth (roller bottles, CellCube®, Cell Factory)

discussed later. The complexity of accessories and systems in this type of equipment is much greater than that in the schematic representation of *Figure 9.5A*. *Figure 9.5B* (see color section) is a photograph of a 300 L stirred-tank bioreactor.

Currently, stirred-tank bioreactors have been scaled-up to working volumes of 20 000 L (Birch and Racher, 2006). Scale-up criteria that take into consideration the negative effects of aeration are generally adopted due to the large influence of bubbling and shear stress on cells. This issue will be discussed in more detail in Section 9.5. Due to the high sensitivity of cells, a limited number of cell lines, such as CHO and NS0, nowadays dominate the industrial animal cell technology arena, due to their greater robustness and shear resistance (Castilho and Medronho, 2002; Castillo *et al*., 2005).

Ensuring aseptic operation is one of the relevant aspects in the operation of animal cell bioreactors. This is a particularly difficult task in large-scale stirred-tank bioreactors where large volumes of fluids are manipulated and moving parts exist. Guaranteeing containment and minimizing the risk of microbial contamination requires careful design of the equipment, a rigorous compliance with strict operational procedures, and the use of simple devices that ensure reliability in the manipulation of large volumes of culture medium.

For the aseptic transport of fluids, arrangements of diaphragm valves especially designed for use in bioreactors and fitted with steam supply for *in situ* sterilization of the whole system (SIP – sterilization in place) are used. Adequate sterilization is guaranteed by online temperature monitoring and the use of pulsing valves with orifices for condensate elimination, among others. The valves are also interconnected with the lines of fluids used for cleaning in place (CIP). Safe mechanical seals or, more recently, magnetic drives, are of great importance to guarantee system containment in spite of the moving parts.

Other elements especially designed to ensure a high efficiency and operational safety are devices that use bellow valves and diaphragm pumps that do not have primary moving parts in contact with the culture medium. More details regarding these and other devices are available in the technical literature (Krahe, 2003).

Validation of systems is obligatory in the production of biopharmaceuticals and, thus, the bioreactors must be ‘‘validatable.’’ In the case of stirred-tank bioreactors, apart from the functional validation, the cleaning and sterilization procedures have to be validated, since these are repeated use equipment. These requirements pose additional challenges to the process engineers.

Wave bioreactors

This type of bioreactor consists of two main parts: a disposable sterile bag made of flexible plastic, in which cells are cultivated, and a moving platform that supports and shakes the plastic bag. A hydrophobic filter attached to the bag allows the supply of gases, and small tubing allows culture sampling, as well as nutrient addition and product harvesting.

The plastic bag is disposable and is provided sterile by the manufacturer. This makes it a very attractive bioreactor type for production purposes, especially if the production scale is not very large. Since it is not sterilized in-house, there is no need for validation of the sterilization procedure. The validation of bag sterilization is the responsibility of its manufacturer. Since it is not reused, there is no need for cleaning and validation of cleaning procedures. *Figure 9.6* (see color section) shows a photograph and a schematic representation of a wave bioreactor.

The oscillatory movement of the platform generates waves in the gas–liquid interface inside the plastic bag. This type of agitation avoids dead zones, maintains cells in suspension and ensures adequate homogeneity, under low shear conditions. Furthermore, the platform can control the culture temperature. The area of the gas–liquid interface, in view of the waves generated by agitation, is several times larger than that of the surface when stationary. This, together with the movement caused in the cell suspension near to the gas interface, significantly increases oxygen transfer. Volumetric oxygen transfer coefficients (K_La) in the range of 3–4 h^{-1} were measured by Singh (1999) for bags with working volumes in the range of 1–100 L, suggesting that cell concentrations up to 7×10^6 cell mL^{-1} could be obtained in this type of bioreactor under nonlimiting conditions with respect to oxygen supply.

However, scale-up of this type of system is limited due to the fact that oxygen transfer is a function of the area of the wave-containing liquid surface. Since the area increases with the square and the volume with the third power of a linear dimension, it is expected that this technology will reach a scale limit. Nevertheless, the companies that market this type of bioreactor offer bioreactors up to at least 500 L working volume (Wave Biotech, 2006).

Recently, approaches to operate wave bioreactors in perfusion mode have been proposed. This turns this type of bioreactor into an attractive variant when there is a need for rapidly establishing a process, on a relatively small scale, and there are no robust facilities for the supply of auxiliary services.

The manipulation of this bioreactor is generally performed in a laminar-flow cabinet, and is thus an open and risky operation. This is probably a characteristic that differentiates this bioreactor type from the other homogeneous bioreactors, where reliability of all seals and connections can be ensured through the use of direct steam. Also, the large number of connections using flexible tubing makes the operation of this type of bioreactor more complicated than that of other bioreactors of the same class. Currently, the performance and application potential of these bioreactors are under evaluation by the biotechnological community.

Air-lift bioreactors

These bioreactors are characterized by their large height-to-diameter ratio and by an internal concentric cylinder, as shown in *Figure 9.7*. In the lower central region, there is a tube that injects gas inside the bioreactor. The pressurized gas stream generates bubbles that result in a low-density region inside the central tube. When these bubbles ascend, the liquid is

pushed upwards through the internal cylinder and returns downwards through the external annulus, generating a characteristic flow pattern, as shown in *Figure 9.7*. Culture oxygenation occurs effectively due to the contact of the cell suspension with ascending bubbles. Furthermore, homogenization of the cell suspension is not due to mechanical agitation, but simply due to the bioreactor geometry combined with the gas stream injected in its bottom region.

These bioreactors are characterized by low levels of cell damage and can also be operated in perfusion mode. However, the main limitation of this type of equipment is related to its scale-up: air-lift bioreactors up to only 5000 L have been described so far.

Air-lift bioreactors were popular in the 1990s, but currently their large-scale use is limited. Only the company Lonza (originally Celltech) uses this type of bioreactor on a large scale, for the production of monoclonal antibodies (mAbs) and other therapeutic proteins (Lonza, 2006).

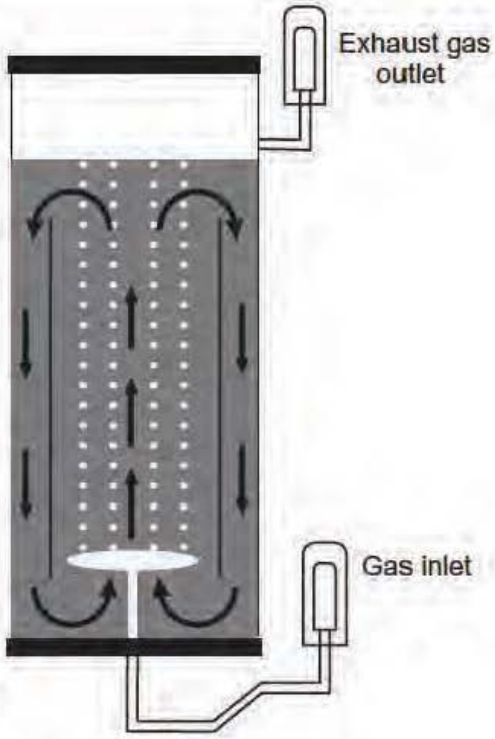


Figure 9.7

Scheme of an air lift bioreactor.

9.3.2 Heterogeneous bioreactors

In this type of bioreactor, there is a compartment where cells remain attached to a surface or immobilized on or inside a biocompatible bed. Culture medium has to be pumped through this compartment for cells to have access to nutrients and dissolved oxygen. The main disadvantages of these bioreactors, developed for the cultivation of adherent cells that require a surface for proliferation, are the impossibility of obtaining homogeneous samples of the cell population and the limitations of scale-up.

Microcarriers

Microcarriers are small particles, made of materials such as cellulose, dextran, glass, collagen, or gelatin. Generally, they have a spherical shape and present a surface structure and composition that promotes cell adhesion and growth.

The first culture of cells on microcarriers was developed by van Wezel in the 1960s (van Wezel, 1967), using DEAE-Sephadex^{TM} 50 gel beads originally designed as ion-exchange chromatography beads. The first product to be produced industrially using microcarriers (an inactivated polio vaccine) was developed by van Wezel himself several years later. Nowadays, the main industrial use of microcarriers is in the production of vaccines, but also of gene therapy vectors, recombinant proteins, and mAbs. In the literature, there are reports of culture scale-up to 6000 L for Vero cells cultivated on Cytodex^{TM} carriers (GE Healthcare, 2006). *Figure 9.8* (see color section) shows Vero cells growing on microcarriers.

The materials used in the manufacture of microcarrriers are porous but, depending on the pore dimensions, the carriers can be classified as macroporous and microporous. The latter, also designated ''solid'' by some authors, have diameters that are too small to allow cells to penetrate the pores. Cell growth, in this case, is restricted to the carrier surface. On the other hand, macroporous microcarriers, also simply designated ''porous'' by some authors, have pores in the range of 10–400 μm, allowing cells to grow inside the pores. Generally, cell concentration inside or on the porous matrices is in the range between 0.5×10^8 and 5×10^8 cells per mL of microcarrier.

Most microporous microcarriers have a bead diameter of 90–300 μm. The cells grow on the surface of the beads, forming an attached monolayer, which may be susceptible to eventual collision of beads and to the resulting mechanical damage. These microcarriers have densities of approximately 1.02–1.04 g cm ^{3}, which is slightly higher than that of the culture medium. Thus, they can be maintained in suspension at low agitation speeds. Furthermore, since their diameter is relatively large, the carriers settle down easily when agitation is stopped, facilitating harvesting of cell-free supernatant.

In the case of macroporous microcarriers, they consist of particles in the range of 0.4–5 mm, which present internal cavities that allow cell growth inside the carriers. This type of microcarrier was developed with the purpose of allowing tridimensional cell growth and attaining high cell densities. The tridimensional growth favors the action of growth factors produced by the cells themselves, decreasing the serum requirements and facilitating the use of serum-free or protein-free media. The high cell concentration makes the cell population more stable and increases culture longevity, making macroporous microcarriers an interesting alternative for long-term cultures. Additionally, their structure protects the cells from shear damage in stirred systems.

There are several manufacturers of microcarriers for animal cell culture, providing products with different chemical composition, size, form, and density. *Table 9.2* shows a list of common commercially available microcarriers and their manufacturers.

Table 9.2 Commercially available microcarriers and their manufacturers

Product	Material	Manufacturer
Microporous microcarriers		
Biosilon®	Polystyrene with negative surface charge	Nunclon
Cytodex™1	Cross linked dextran with DEAE groups	GE Healthcare
Cytodex™3	Cross linked dextran coated with a denatured collagen layer	GE Healthcare
HyQ® Sphere™	Polystyrene with different options of charge and coating	Hyclone/ SoloHill
Macroporous microcarriers		
Cultispher®	Cross linked porcine gelatine	Percell Biolytica
Cytoline™	Polyethylene/silica	GE Healthcare
Cytopore™	Cross linked cellulose with DEAE groups	GE Healthcare

When anchorage-dependent cells are immobilized by microcarriers they can be cultivated in homogeneous bioreactors (Jo *et al*., 1998). Furthermore, the immobilization on the carriers allows the use of other bioreactors that will be discussed in the following sections, such as fluidized-bed and packed-bed bioreactors. The microcarriers can be separated from the supernatant by sedimentation, facilitating downstream processing or cell retention in perfusion processes.

However, the use of microcarriers on a large scale presents some problems regarding inoculum propagation. It may be desirable that cells already attached to microcarriers used at an intermediate scale colonize new carriers to inoculate a larger-scale culture. Enzymatic and sonication methods can be used to release cells from microcarriers, but the conditions can be harsh to the cells and present negative effects on cell physiology. A more recent approach and an alternative to the total release of cells, is bead-to-bead transfer. This technique seems to be highly dependent on the combination of adhering characteristics of the cells and properties of the microcarriers. The efficient transfer of cells between particles is only possible with some cell–carrier combinations (Wang and Ouyang, 1999).

Furthermore, the homogeneity of parameters and the mass transfer of nutrients to the interior of macroporous microcarriers can be critical, especially for larger beads and at larger cultivation scales (Rupp, 1987).

Packed-bed bioreactors

In packed-bed bioreactors, cells are maintained in a fixed bed, that is, confined in a given environment. In most applications, cells adhered to microcarriers are used in these bioreactors. Culture medium is recirculated through the packed bed, promoting transfer of nutrients, metabolites, and products. Before being pumped into the bed, the medium should be enriched in dissolved oxygen. This can be accomplished by vigorous stirring in aeration tanks or by the use of membrane modules, generally positioned in an external recirculation loop. The size of the packed bed is a critical parameter, since the dissolved oxygen concentration in the medium

gradually decreases as the liquid flows through the bed (Fassnacht and Porter, 1999).

One of the advantages of this system is that a clarified fluid is obtained at the bioreactor outlet, with no need for a cell separation device. *Figure 9.9* (see color section) shows the operational scheme of a fixed-bed bioreactor and a photograph of a small-scale model.

Fluidized-bed bioreactors

Fluidized-bed bioreactors are also used mainly for the culture of cells attached to microcarriers. However, in this case, microcarriers with a higher density should be employed. In these bioreactors, a given amount of microcarriers is fluidized inside a compartment of the bioreactor, avoiding cell damage due to bubble explosion. *Figure 9.10* (see color section) shows a photograph and an operational scheme of this type of bioreactor.

These bioreactors are operated in perfusion mode and cell concentrations reach 5 to 10 million cells per mL of medium. Several authors report their results in terms of cell concentration per microcarrier volume and, in this case, values in the order of 20 to 35 million cells per mL of carrier are obtained (Wang *et al*., 2002).

Bioreactors providing surfaces for attached cell growth

Roller bottles and systems with multiple plates, such as Cell Factory (Nunc) and CellCube® (Corning), provide a large surface area for cell adhesion and proliferation. In the case of roller bottles, although the relative surface area (area/volume) is small and consequently the production capacity is low, large-scale plants can be established by using a large number of roller bottles. This approach was adopted by the company Amgen for the industrial production of erythropoietin. Operation costs for this type of plant are high, because they are labor-intensive, and the risk of contamination is high, due to the manipulation of lots of small bottles.

On the other hand, the principle of CellCube® and Cell Factory is to provide a large area for cell adhesion and proliferation in relatively compact devices. The latter consists of multiple trays, providing surface areas in the range of 700–25 000 cm^2, for medium volumes of 0.2–8 L (Nunc, 2006). The system called CellCube® provides a surface area in the range of 8500–85 000 cm^2 and is fitted with an automated control system (Corning, 2006).

Hollow-fiber bioreactors

The core part of a hollow-fiber bioreactor is the hollow-fiber membrane module, also simply known as the cartridge. It consists of a plastic cylinder containing hundreds of semi-permeable capillary tubes, known as hollow fibers. The cells are inoculated in the extracapillary space (ECS). The cells colonize the external surface of the fibers and grow in this region. The culture medium is pumped through the lumen of the fibers, known as the intracapillary space (ICS), as shown in *Figure 9.11*.

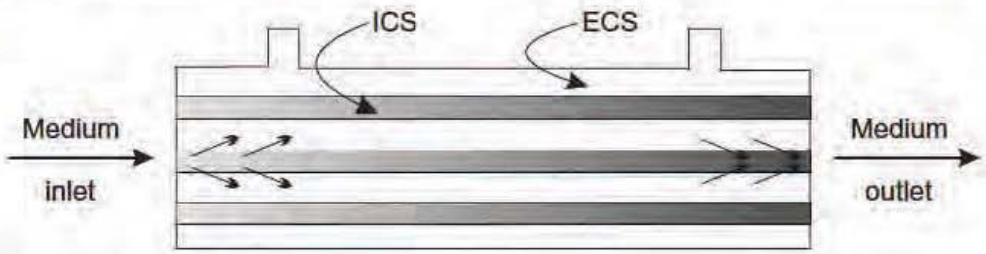


Figure 9.11

Simplified representation of a hollow fiber module with only three fibers. The straight arrows pointing from the intracapillary space (ICS) to the extracapillary space (ECS) represent the transfer of nutrients from the culture medium to the cells (inoculated in the ECS), whereas the straight arrows in the opposite direction represent the transfer of metabolites and products from the cells to the fiber interior.

The fibers can be made of different materials, such as cellulose esters and polysulfone. The total surface area of a hollow-fiber bioreactor varies in the range of 0.5–3.5 m^2. The pore size of fibers commonly employed in animal cell culture corresponds to a molar mass cut-off between 10 and 100 kDa.

The porous nature of the fibers allows for exchange of nutrients and metabolites. Low-molar-mass molecules, such as glucose and ammonia, can move freely through the pores of the fibers, at a rate that is controlled just by the pressure gradients generated by the medium recirculation pump. High-molar-mass proteins, which can be produced by the cells or added as nutritional supplements to the extracapillary space, are not able to permeate the membrane fibers and are retained in the cell bed in the ECS.

Thus, the cell population grows in the ECS of the hollow-fiber cartridge, receiving the nutrients originating from the culture medium that is recirculated through the interior of the fibers. Inhibitory metabolic products, generated as a consequence of cell growth, diffuse in the opposite direction and get diluted in the recirculating culture medium. In the same way, low-molar-mass proteins and growth factors produced by the cells or provided by serum can permeate the fibers. Thus, in the axial direction of the fibers a decreasing gradient of medium components and an increasing gradient of metabolites are formed. This process may lead to a decrease in the transfer of nutrients to the microenvironment of the cells, with the possibility of the concentration of these molecules becoming growth-limiting. This may become critical when fiber length is increased, since a region very poor in nutrients and very rich in inhibitory metabolites can be created in the final portion of the fibers. This situation can be attenuated through the introduction, directly through the ECS, of an additional stream perpendicular to the fibers, known as recycle stream. Culture medium containing serum can be added to this recirculation stream, while basal medium is recirculated through the lumen of the fibers. *Figure 9.12* shows the scheme of a hollow-fiber bioreactor fitted with an extracapillary recirculation line.

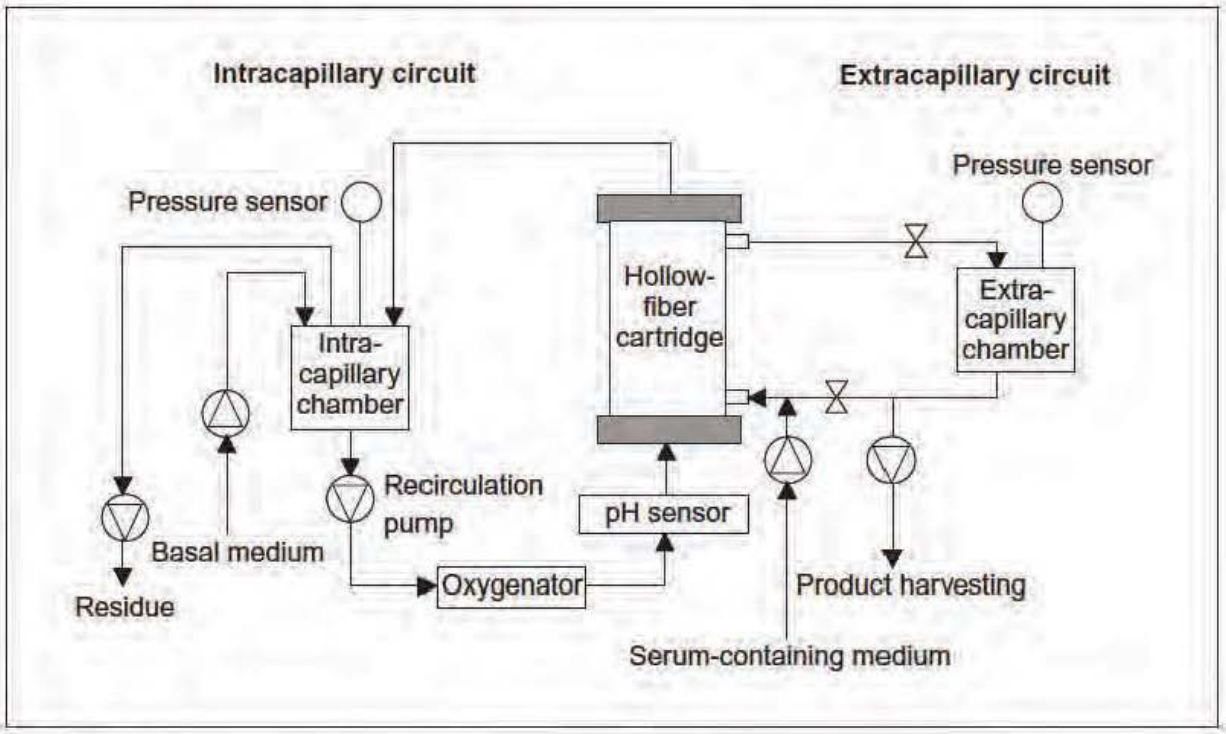


Figure 9.12

Schematic representation of a hollow fiber bioreactor fitted with an extracapillary recirculation stream.

Oxygen transfer is an important issue in the operation of a hollow-fiber bioreactor. In these bioreactors, a membrane-based aeration system is usually included in the intracapillary recirculation loop to enrich the recirculating culture medium with oxygen. However, due to the low solubility of oxygen in aqueous solutions, the recirculation speed through the fibers must be very high.

It is possible to modify some design parameters to determine optimal operational conditions for these bioreactors, such as the fiber material, the packing density of fibers inside the module, the dimensions of the module, the pore size of the fibers, and the type of aeration system used in the recirculation loop. Furthermore, the yield of these bioreactors can also be improved by optimizing the operational strategies, such as: implementation of an extracapillary recirculation loop; increase of the flow rate through the oxygenation device; execution of periodic cell purges from the ECS, to avoid culture necrosis; use of an adequate serum content or of an improved medium composition to favor cell proliferation; and determination of an optimal time and scheme for the extracapillary recirculation.

Parameters that are traditionally used to evaluate the metabolic activity of the cell population inside a hollow-fiber bioreactor include glucose consumption rate (r_{GLC}) and oxygen consumption rate (r_{O2}). However, these parameters are highly dependent on variations in nutrient feed and metabolic products removal (Gramer *et al.*, 1999). The use of a strategy to overfeed the ICS with nutrients results in a rapid increase in r_{GLC}. On the other hand, an increase in product titer and an improved metabolic

efficiency in terms of product formation can be attained if an operation strategy is adopted that includes a perpendicular extracapillary recirculation stream and a regular purge of cells, to operate at lower biomass concentrations (Rodríguez *et al.*, 2005).

In a study of the production of a humanized mAb produced by NS0 cells, Rodríguez *et al.* (2005) compared two different operation strategies (*Figure 9.13*). The optimized strategy provided a significant decrease in the ratio of the flow rates of product harvest and medium feed, with a twofold increase in the total amount of antibody harvested and a sixfold decrease in the harvested volume. Thus, antibody concentration in the product stream was increased by over 10-fold, with a positive impact on the purification process of this mAb.

9.4 Modes of operation of bioreactors

The different operation modes used in microbial fermentations are employed also in animal cell cultivation. Although many different classifications can be adopted, the most general is the one that considers the following operation modes: batch, fed-batch, continuous, and perfusion, which is a continuous mode with cell recycle/retention (Castilho and Medronho, 2002).

The behavior of a culture system in terms of cell, nutrients, and product concentrations can be described mathematically by mass balances and

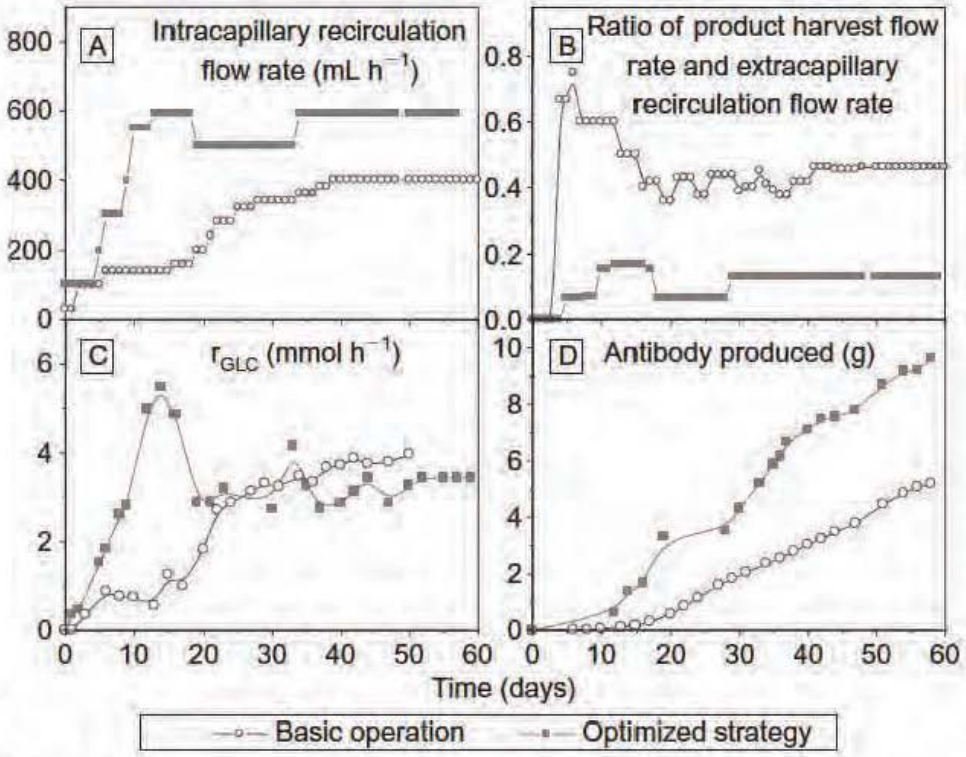


Figure 9.13

Cultivation of NS0 cells in an AcuSyst Jr™ hollow fiber bioreactor: comparison of two different operation strategies, designated basic and optimized strategy (Rodríguez *et al.*, 2005).

kinetic expressions, such as the Monod equation, which describe the effects of nutrient and metabolite concentrations on the specific reaction rates.

In order to make a decision on the most appropriate operation mode for a bioreactor, several factors must be taken into consideration. At industrial scale, the most important factors are:

(i) Characteristics of the cell line used, such as the stability of expression of the product, production pattern, degree of resistance to inhibitory metabolites, and resistance to shear stress;
(ii) The market demand for the product, which determines the production scale;
(iii) The technical experience of the teams responsible for both process development and regulatory issues.

9.4.1 Batch cultivation

Batch is a discontinuous operation mode, and cell growth occurs without any additional supplementation of nutrients after inoculation of cells. While substrates are metabolized, the cell population grows, forming the product and other metabolites. The volume is maintained constant throughout the whole process.

Equations 1 to 3 represent the generic mass balances for cells, substrate and product in a batch cultivation process:

$$\frac{dX_v}{dt} = \mu X_v \tag{1}$$

$$\frac{dS}{dt} = -q_S X_v \tag{2}$$

$$\frac{dP}{dt} = q_P X_v \tag{3}$$

Where:
X_V = viable cell concentration;
t = cultivation time;
μ = specific growth rate;
S = concentration of a generic substrate;
q_S = specific consumption rate of a generic substrate;
P = concentration of a generic product;
q_P = specific formation rate of a generic product.

Figure 9.14A shows the operational configuration and typical profiles for cell and substrate concentration in a batch culture. *Figure 14B* shows the concentration of cell, product (mAb), and substrates (glucose and glutamine) for a myeloid transfectoma in batch cultivation.

In a batch cultivation, cell concentration increases from the inoculation density (0.1–0.4 million cells per mL) up to maximum values between 2 and 4 million cells per mL, being the upper limit applicable to insect cell cultivation. Since animal cells present specific growth rates between 0.02 and 0.04 h^{-1}, the time period to attain maximum cell density in a batch

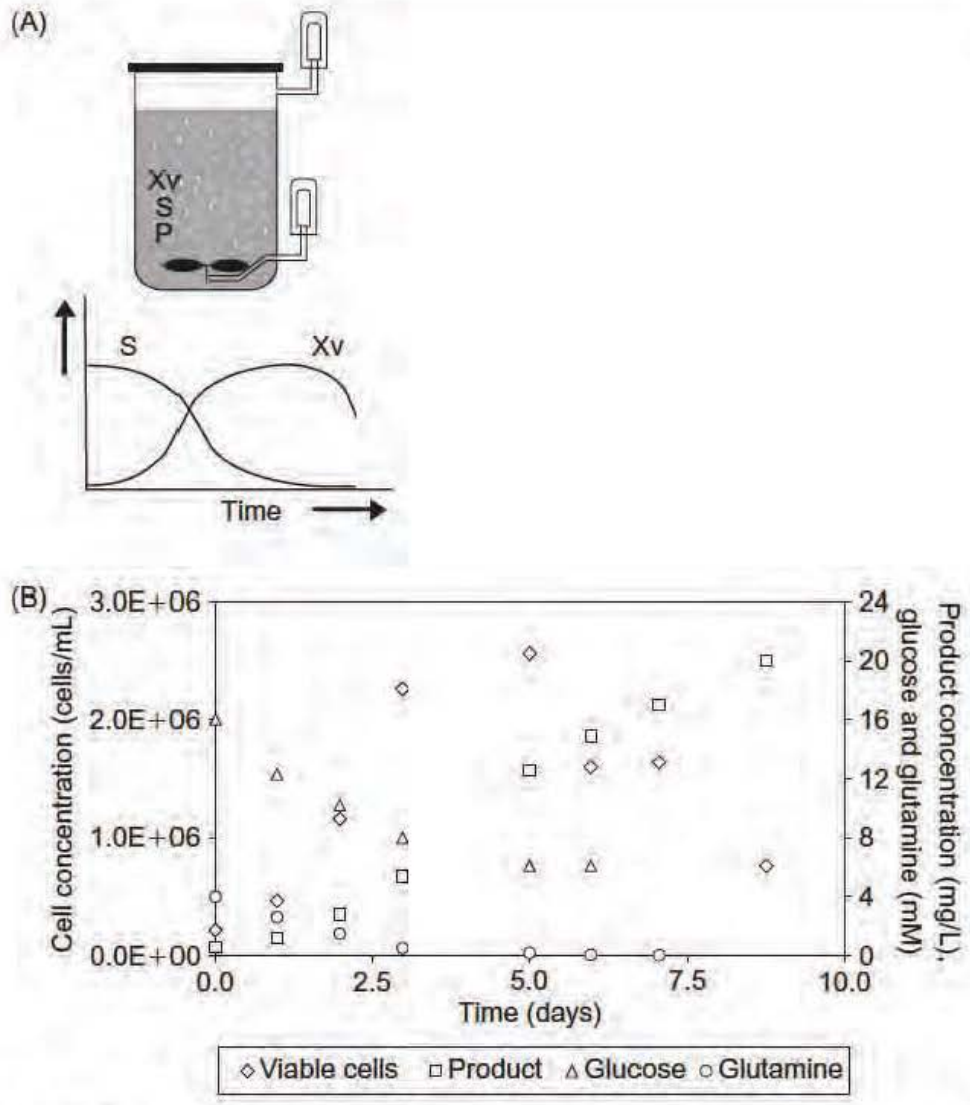


Figure 9.14

Batch cultivation: (A) operational configuration and typical viable cell (Xv) and substrate (S) concentration profiles; (B) concentration of cells, product and two substrates in a batch bioreactor culture of a myeloid transfectoma producing a humanized monoclonal antibody (Center of Molecular Immunology CIM, Cuba).

culture is approximately 5 days. The range of specific product formation rates is wider, varying from a value as high as 30 pg cell ^{1} day ^{1}, reported for mAb production by transfectomas (Velez *et al.*, 1987), to values two orders of magnitude lower (Altamirano *et al.*, 2004). The concentration of a secreted product in batch cultures is usually in the range of 1–50 mg L ^{1}. After attaining maximum product concentration, the culture is interrupted and the spent medium is sent to the downstream processing stages.

Due to the low solubility of oxygen, this gas must be supplied continuously, instead of just at the start of the culture, as in the case of the other nutrients. Control of pH is carried out through addition of base and by varying the CO_2 concentration in the gas phase, since most animal cell culture media contain sodium bicarbonate.

The batch operation mode is the simplest to carry out, and therefore it is widely employed. Generally, it is adopted for the cultivation of cells in stationary flasks, in stirred flasks, in small- and intermediate-scale bioreactors for inoculum propagation and also in several industries at production scale. In the case of inoculum propagation, the culture is stopped when the cells are still in the exponential growth phase, to provide a high mass of cells growing at maximum rate. In this way, it is guaranteed that these cells, when inoculated in a larger bioreactor, will present a minimal lag phase, and so reducing the non-productive period.

The low volumetric productivities that characterize batch cultivation processes are a disadvantage for the use of this operation mode for production. However, a variant known as ''repeated batches'' is an interesting alternative. It consists of initially carrying out a batch cultivation for the time needed to attain the desired product concentration. At that moment, just a part of the bioreactor contents is harvested. The remaining cell suspension inside the bioreactor is then used as inoculum for a new batch, by filling the vessel with fresh medium. This procedure can be repeated several times, until a decrease in cell growth or product formation is observed. The use of repeated batches allows a decrease of the time the bioreactor is non-productive. This eliminates the time periods that would be necessary for cleaning and sterilization between each batch.

The productivity of batch cultures in general is limited by the low cell concentration and, consequently, the low product concentration at the end of the process. This is related to the fact that the initial concentration of substrates must be relatively low to avoid cell death due to osmotic effects (Morbach and Krämer, 2003), as well as to the fact that the cells are submitted to environmental conditions that keep changing during the whole process.

The main technological features of the batch operation mode are:

(i) operational simplicity;
(ii) simple equipment and operational procedure, decreasing contamination risks, when compared with other operation modes;
(iii) low productivity, that can be improved slightly by carrying out repeated batches;
(iv) process optimization possible through manipulation of medium composition and determination of the best time to harvest the product;
(v) applied in industrial processes at scales up to 20 000 L.

9.4.2 Fed-batch cultivation

The difference of this operation mode compared with batch is that one or more nutrients are fed during cultivation, to replace those consumed by the cells. Fed-batch cultures begin with a working volume lower than the maximum working volume, so that nutrients can be added during the culture period, either from fresh culture medium or from a concentrated nutrient solution. *Figure 9.15A* shows typical profiles for cell and product concentrations, as well as the volume of medium in the reactor resulting from pulse additions of nutrients.

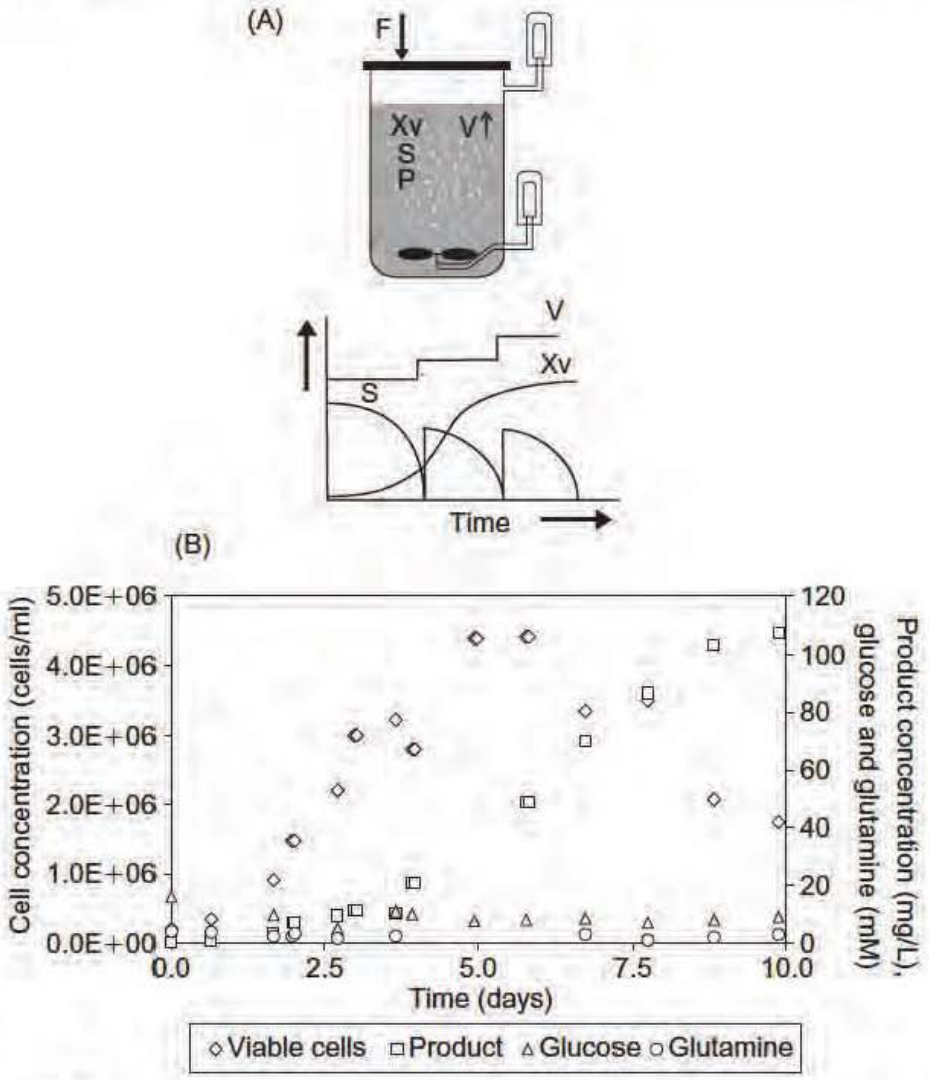


Figure 9.15

Fed batch cultivation: (A) operational configuration and typical volume, cell, and substrate profiles, for a pulse feeding strategy; (B) concentration of cells, product, and two substrates in a fed batch bioreactor culture of a myeloid transfectoma producing a humanized monoclonal antibody (Center of Molecular Immunology CIM, Cuba).

Feeding nutrients during the culture allows an increase of the total amount of nutrients metabolized by the cells, which results in higher final concentrations of cells and product. Effects of high initial nutrient concentrations can be eliminated, since the nutrients are not added all at once at the beginning of the process.

Equations 4 to 6 present the mass balances for cells, substrate, and product in a fed-batch culture:

$$\frac{dX_v}{dt} = \mu X_v - \frac{F}{V} X_v \tag{4}$$

$$\frac{dS}{dt} = -q_s X_v + \frac{F}{V}(S_A - S) \tag{5}$$

$$\frac{dP}{dt} = q_P X_v - \frac{F}{V} P \quad (6)$$

Where:
F = feed flow rate, which usually varies with time;
S_A = concentration of a generic substrate in the feed stream;
V = bioreactor working volume, which varies with time.

As can be seen, these equations differ from those presented for batch cultures, since they contain terms related to the addition of nutrients from a solution with concentration S_A at a flow rate F.

Figure 9.15B shows the behavior of a fed-batch culture. Starting from the inoculum concentration, a cell density of 5×10^6 cells mL ^{1} is obtained within a period of 7–10 days, although in the literature higher concentrations (10×10^6 cells mL ^{1}) have been reported (Xie and Wang, 1997). Furthermore, in some cases, the cultivation time can be extended to 10–15 days. The most relevant in this context is that product concentration can be increased significantly, when compared with batch cultivation. This is evidenced by Equation 6 (mass balance for product), taking into consideration that values for cell mass and culture times are larger in this case than in batch cultures. Indeed, some of the final concentration values reported for this operation mode are among the highest titers attained for animal cell products. Conventional product concentrations of 150 mg L ^{1} are usually achieved in fed-batch cultures, but after optimization of feeding strategies and using improved recombinant cell lines, Robinson (1994) reported product concentrations of approximately 2 g L ^{1}.

Nutrient feeding can be carried out according to different strategies; addition of nutrients in pulses, in steps, at constant flow rate, or using more sophisticated strategies, such as an exponentially growing feed flow rate. By using optimized strategies, based on the specific consumption rates for each substrate and on the effect of nutrient concentration on these rates, cell concentrations up to 2.5-fold higher than those observed in batch mode were achieved by Xie and Wang (1997).

Another interesting positive effect that has been observed in fed-batch cultivation is the duration of the stationary phase (Altamirano *et al*., 2004). When cells show non-growth associated or inversely associated product formation kinetics, the prolonged stationary phase observed in fed-batch cultures results in considerable gains in productivity. The use of molecular biology tools to change the production pattern of a cell line for a non-growth associated pattern aims at exploiting this advantage (Weikert *et al*., 1999).

In fed-batch culture processes, productivity is usually limited by the accumulation of toxic metabolites, which can inhibit cell growth and negatively influence culture viability and product formation. The most relevant toxic metabolites are lactate and ammonia (Hassell *et al*., 1991). Feeding strategies aimed at maintaining low levels of nutrients, as well as total or partial substitution of glucose and glutamine by slowly metabolized substrates, allow a decrease of the level of lactate and ammonia in cultures. In a fed-batch culture of a tPA-producing CHO cell line, Altamirano *et al*. (2004) proposed replacing glucose by galactose, a more

slowly metabolized sugar, and this caused a decrease in the generation of lactic acid, as well as an increase in cell viability and product formation.

Another active research field is the optimization of intracellular metabolic reactions to decrease the production of toxic metabolites through metabolic engineering strategies. An example is the introduction of the glutamine synthetase (GS) gene into NS0 cells, that do not have this activity, or into CHO cells, that have a low endogenous GS activity. GS catalyzes the formation of glutamine from glutamate, allowing cells to grow in a medium with low concentrations of glutamine or no glutamine at all. The introduction of this gene leads to a considerable decrease in ammonia levels in the cultures, resulting in a significant increase in maximum cell concentration and prolonged duration of the stationary phase. As a consequence, final product concentration and volumetric productivity are increased (Bebbington *et al*., 1992). This approach has been applied industrially for both cell lines – NS0 and CHO.

A disadvantage of fed-batch culture is the long residence time of the product in the culture environment. A product molecule, secreted at the early phase of the process, remains inside the bioreactor for the whole duration of the culture, in the presence of proteases and glycosidases (Goochee and Monica, 1990). Since the product of interest is generally a protein (usually a glycoprotein), these enzymes may degrade the product and, in some cases, destroy an important fraction of the active material that is synthesized. For that reason, molecules that are unstable at the culture temperature or prone to enzymatic cleavage should not be produced by fed-batch cultures.

The most important characteristic of the fed-batch mode is the fact that, in spite of its more complex operation compared with batch cultures, the productivity is increased significantly. Furthermore, when compared with perfusion operation, the runs are considerably shorter, making process validation easier. For this reason, a relatively large number of biotechnological companies have developed their production processes based on this technology (Chu and Robinson, 2001).

In summary, the main technological features of this operation mode are:

(i) moderate operational complexity;
(ii) moderate contamination risks;
(iii) intermediate volumetric productivity;
(iv) long residence time of product in the bioreactor;
(v) process optimization possible basically through manipulation of medium composition, feeding strategy, and harvesting time;
(vi) applied in industrial processes at scales up to 20 000 L.

9.4.3 Continuous cultivation

Continuous cultivation is used mainly in research and development activities, at small scales. It is characterized by a continuous feed of fresh medium and a continuous removal of cell suspension, both at the same flow rate, and at constant bioreactor volume.

Equations 7 to 9 describe the behavior of a continuous culture:

$$\frac{dX_v}{dt} = \mu X_v - DX_v \tag{7}$$

$$\frac{dS}{dt} = -q_S X_v + D(S_A - S) \tag{8}$$

$$\frac{dP}{dt} = q_P X_v - DP \tag{9}$$

Where:
D = dilution rate (D = F/V).

When the system reaches the steady state, concentrations of cells, product, and substrates remain constant, so that according to Equation 7 the specific growth rate becomes equal to the dilution rate.

Figure 9.16A shows a typical operational configuration of a continuous bioreactor culture. In *Figure 9.16B*, the cell concentration data of a real continuous culture, operated at three different dilution rates, are presented. The rectangles indicate the different steady states observed, whereas the arrows show the moment of change in dilution rate. Furthermore, a guide line has been included in *Figure 9.16B* to help interpretation of the cell concentration profile.

The continuous mode allows the establishment of well-defined steady states, so that the relationship between the concentration of substances inside the bioreactor and different biological reaction rates can be studied. Therefore, this operation mode is a powerful tool for cell characterization (Altamirano *et al*., 2001).

When carrying out studies in continuous mode, it is important to guarantee that the system stabilizes before measurements are made, to ensure that reliable results are obtained. The determination of physiological parameters in non-steady state can be influenced by effects of adaptation or transition of the biological system, therefore making measurements imprecise (Vits and Hu, 1992).

A limiting condition when carrying out continuous culture is the situation known as bioreactor wash-out. This condition is characterized by a cell removal rate at the bioreactor outlet that is equal to or higher than the cell growth rate, such as that shown in *Figure 9.16B* after D has been increased to 0.8 d^{-1}. The wash-out condition occurs when the dilution rate is increased to a threshold value called $D_{wash\ out}$, which can be derived from Equation 7, considering steady state and adopting μ_{MAX} for μ.

$$\mu_{MAX} = D_{wash\ \ out} \tag{10}$$

Dilution rate values higher than $D_{wash\ out}$ cause a progressive decrease in cell concentration, until no more cells are found inside the bioreactor. Thus, $D_{wash\ out}$ is the upper limit that should not be exceeded in a continuous culture. Since the μ_{MAX} values for animal cells are between 0.02 and 0.04 h^{-1} (0.5–1 d^{-1}), the maximum cell concentration usually does not surpass 2×10^6 cell mL^{-1}. Considering that productivity in a contin-

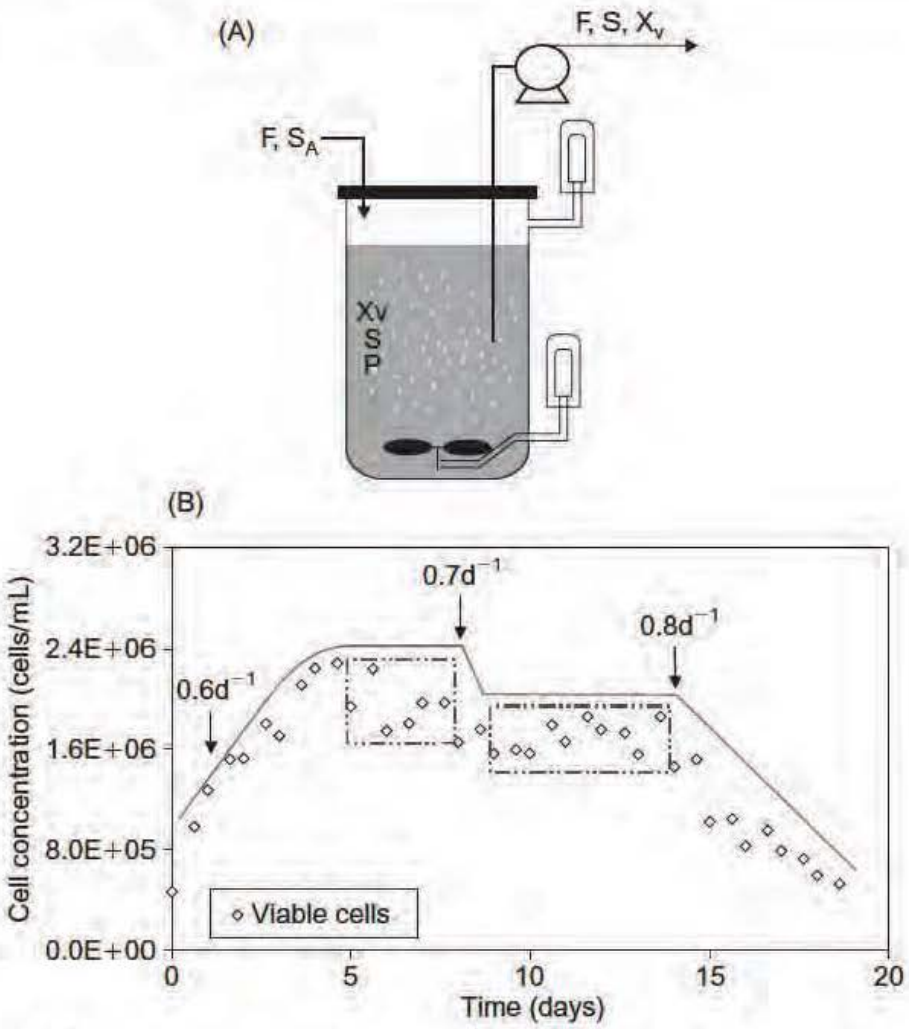


Figure 9.16

Continuous cultivation: (A) typical operational configuration; (B) cell concentration as a function of dilution rate for a continuous culture of a myeloid transfectoma producing a humanized monoclonal antibody (Center of Molecular Immunology CIM, Cuba). The different steady states are indicated by rectangles and the line has been included to facilitate interpretation of the cell concentration profile.

uous process can be expressed by multiplying the dilution rate by the product concentration (DP), it is easy to conclude that low cell concentrations result in low product concentrations and low productivities, making this process not very attractive for industrial applications.

9.4.4 Continuous cultivation with cell retention (perfusion)

Widely known as perfusion, this operation mode presents the highest productivities and, at the same time, the highest operational complexity. Since the innovating work on a spin-filter in 1969 (Himmelfarb *et al.*, 1969), the use of this operation mode has become more and more popular, both at laboratory and industrial scales (Chu and Robinson, 2001).

In this operation mode, it is possible to mitigate the major limitation of continuous cultures, that is, the low productivity due to the loss of cells in the bioreactor outlet. In perfusion, this issue is overcome by using a cell retention device to maintain cells inside the bioreactor. *Figure 9.17* shows a scheme of a stirred-tank bioreactor operating in perfusion mode, as well as the kinetic behavior of a perfusion run.

Equations 8 and 9, previously shown for the substrate and product mass balances in continuous cultures, can also be applied to this cultivation mode. However, due to cell retention, the equation that expresses the cell mass balance is altered, when compared with the mass balance of cells in a continuous culture (Equation 11):

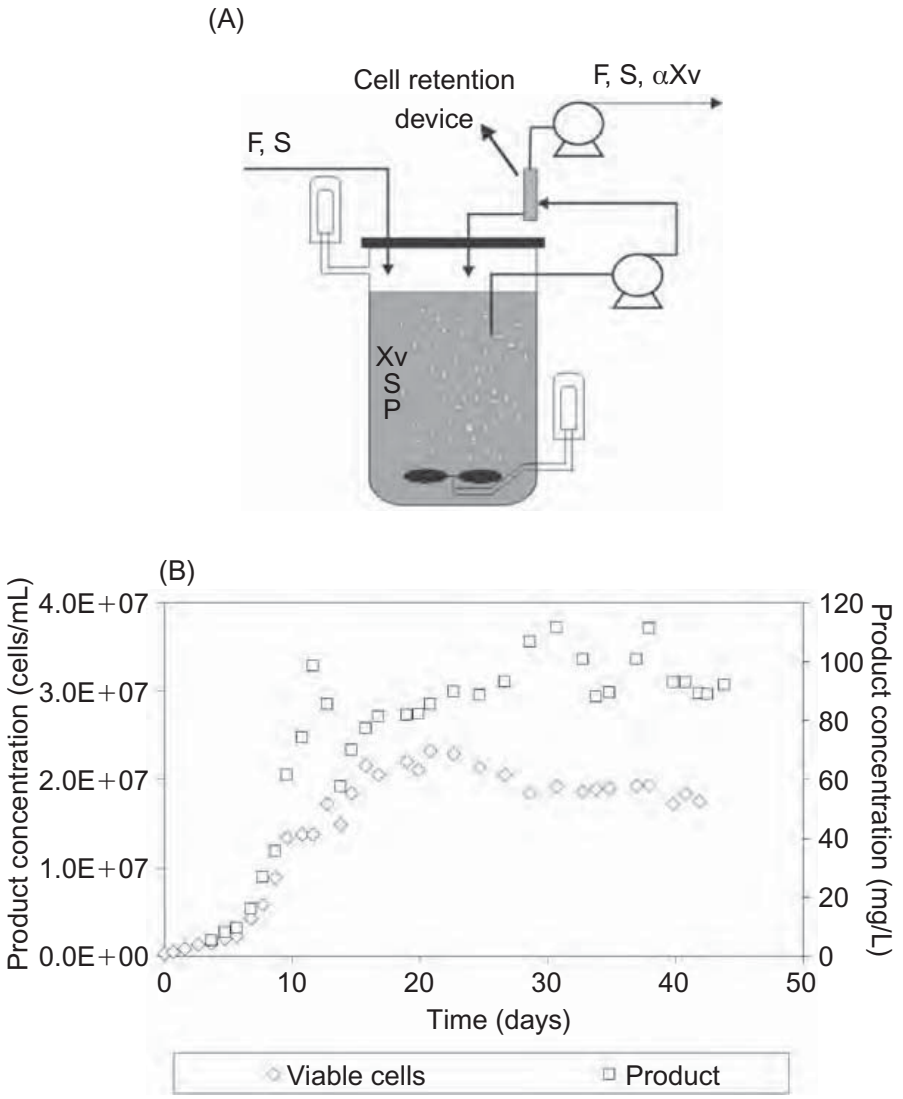


Figure 9.17

Perfusion culture: (A) typical operational configuration, using an external cell retention device; (B) concentration of viable cells and product in a perfusion bioreactor culture of a myeloid transfectoma producing a humanized monoclonal antibody (Center of Molecular Immunology CIM, Cuba).

$$\frac{dX_v}{dt} = \mu X_v - \alpha D X_v \tag{11}$$

In Equation 11, α is the cell passage factor, defined as the ratio between cell concentration in the perfusate and in the bioreactor (Equation 12).

$$\alpha = \frac{X_{v,PERFUSATE}}{X_v} \tag{12}$$

Cell retention may be total or partial. A total cell retention would result in $\alpha = 0$, whereas a value of 1 would mean no retention of cells.

Wash-out conditions in perfusion cultures are different due to the retention of cells. In the same way as Equation 10 was obtained, Equation 13 can be derived from Equation 11:

$$\mu_{MAX} = \alpha D_{wash\ \ out} \tag{13}$$

For low α-values (between 0 and 0.2), the dilution rate may be increased up to values that are much higher than the maximum specific growth rate, without establishing a wash-out condition. The increase in the dilution rate that can be employed results in a greater availability of nutrients and, consequently, in an increase of cell and product concentrations. Thus, the perfusion mode allows a high-cell-density culture to be maintained for a long period, at a high perfusate flow rate and an elevated product concentration. This results in productivities that are 1–2 orders of magnitude higher than those obtained in continuous processes. In fact, the performance of perfusion cultures is superior to that of all other operation modes (Bibila and Robinson, 1995).

The operation of cultures in perfusion mode is possible for almost all existing bioreactor types. Heterogeneous bioreactors are usually operated in perfusion mode, and homogeneous bioreactors can be if a solid–liquid separation device (cell retention device) is used (see Chapter 11).

As indicated for other modes, it is necessary to maintain the inoculum concentration in the range of 0.1×10^6 to 0.4×10^6 cells mL^{-1}, to minimize the adaptation lag phase at the beginning of a cultivation process. The profiles for cell growth and product formation will depend on the feeding strategy adopted, on the cell line characteristics, and on the performance of the cell retention device.

In the case of homogeneous bioreactors, the maximum cell concentration in perfusion cultures can attain 10^7–10^8 cells mL^{-1}. When this operation mode is used in heterogeneous bioreactors, cell concentration in the cell compartment can approach the packing limit of tissues, which is in the order of 10^9 cells mL^{-1}. Product concentrations reported for these processes vary considerably, but are most commonly in the range of 100–500 mg L^{-1}. Culture duration can be in the range of several days up to several months (Bödeker, 1994).

The feeding strategy adopted in a perfusion process is of great importance. The ideal approach, that is, the one that results in the highest productivity, depends on the relationship between growth kinetics and product formation kinetics. For cell lines that present growth-associated production, it is recommended to feed culture medium at an increasing dilution rate to allow maximum cell growth. However, if product forma-

tion by the cells is not associated or inversely associated with growth kinetics, then the dilution rate should be increased up to a given value and then remain constant, resulting in a steady state of the main process parameters (Figueredo, 2002).

An example of the first strategy is the so-called cell-specific perfusion rate (Ozturk, 1996), which allows maintenance of cells in a constant environment by exponentially increasing the feed rate according to the cell concentration profile (Chico and Jäger, 2000). A negative aspect of this strategy is that very high cell concentrations are achieved rapidly, and this may negatively affect the cell separation device (Deo *et al*., 1996), and thus limit the culture duration. This negative aspect can be minimized by establishing a cell bleed stream for a controlled removal of cells from the bioreactor. This allows maintenance of a high cell viability, combined with a stable and high cell concentration, at a level that is compatible with good performance of the cell separation device. Furthermore, the high dilution rates used in this case result in short residence times of the product inside the bioreactor, which can be relevant in the case of products that are very unstable or that can be easily degraded under culture conditions.

Another feeding strategy, which is sometimes called ''stationary strategy'', aims at extending the culture duration for months (Bödeker, 1994). However, the use of this type of strategy results in a considerable decrease in cell growth rate (Figueredo, 2002) and in an accumulation of dead cells, causing the release of cell debris and substances potentially dangerous to product integrity. To diminish this problem, cell purges are periodically carried out in order to maintain a more constant and controlled cell environment (Kempken *et al.*, 1991).

Although in most cases perfusion operation presents several economic advantages, its industrial use is not as widespread as that of fed-batch cultures. This is due mainly to false ideas of the technological and operational difficulties and an underestimation of the productivity gains of this operation mode. A critical evaluation of the characteristics of perfusion operation indicates that the disadvantages have usually been emphasized or even exaggerated (Kadouri and Spier, 1997).

The design of bioreactors for perfusion operation is more sophisticated, which makes the equipment more expensive. However, the productivity increases obtained by perfusion operation allow the use of much more compact systems than those operated under batch or fed-batch mode. In this way, perfusion bioreactors can be up to 10-fold smaller for a given production scale (Bibila and Robinson, 1995), decreasing the costs not only of the bioreactors themselves, but also of storage tanks and downstream processing equipment.

As regards validation, perfusion bioreactors, which are more complex and of more recent use, have been classified in the past as difficult to validate (Kadouri and Spier, 1997). The longer cultivation runs result in longer validation processes when compared with discontinuous processes. However, the growing number of approved biopharmaceutical production processes based on perfusion mode suggests a change in attitude by the regulatory agencies (Chu and Robinson, 2001).

In summary, the main technological features of this operation mode are:

(i) more complex operation than in the case of other operation modes;
(ii) higher contamination risk, since it is an open system that operates continuously for long periods of time;
(iii) higher volumetric productivity;
(iv) short residence time of product in the bioreactor (ideal for the production of labile molecules);
(v) process optimization basically through manipulation of medium composition, feeding strategy, and controlled cell removal (cell bleeding);
(vi) scales generally up to 2000 L.

9.5 Aeration and agitation

Aeration and agitation are two important operations in animal cell culture. Oxygen has a low solubility in aqueous media, with a saturation concentration of approximately 7 mg L^{-1} at 37°C. This implies that the oxygen must be provided continuously to the cultures, to ensure that the dissolved oxygen levels in the culture medium remain at an adequate level. The mass balance for oxygen in the liquid phase can be written as:

$$\frac{d(DO)}{dt} = k_L a\,(DO^* - DO) - q_{O2} X_v \tag{14}$$

Where:
DO^* = concentration of dissolved oxygen at saturation (in equilibrium with the gas phase);
DO = dissolved oxygen concentration;
k_L = global oxygen transfer coefficient;
a = gas–liquid interfacial area per reactor volume;
q_{O2} = oxygen specific consumption rate.

This mass balance concerns the liquid phase, since oxygen must be dissolved in order to be used by the cells. Due to the difficulty in measuring the interfacial area (a), especially when oxygenation is carried out by bubble aeration, it is common to use the product of k_L times a ($k_L a$), known as the volumetric oxygen transfer coefficient, as the relevant parameter.

The dissolved oxygen concentration at equilibrium follows Henry's Law (Equation 15), with the constant H_{O2} being a function of temperature. Since the air contains 21% of oxygen, the partial pressure P_{O2} when using pressurized air is fivefold lower than when using pure oxygen. Thus, DO^* is higher for pure oxygen injection, resulting in a larger oxygen transfer rate to the liquid medium.

$$DO^* = H_{O_2} \cdot P_{O_2} \tag{15}$$

Where:
P_{O2} = oxygen partial pressure;
H_{O2} = Henry's constant.

Different studies have established an optimal dissolved oxygen concentration for animal cell culture in the range of 20–50% of air saturation

(Butler, 2004). Too high oxygen concentrations lead to the formation of free radicals in the medium, which may cause oxidative damage to cells. The DO concentrations are monitored with previously calibrated electrodes, whereby the 100% value is adjusted after saturation of culture medium with air, at the cultivation temperature (usually 37°C). The oxygen transfer system must be designed to meet the demands of the culture, avoiding situations of limitation or excess of dissolved oxygen. This is accomplished by supplying air, pure oxygen, or mixtures of both gases to the culture. Another aim of aeration is to allow dissolved carbon dioxide originating from cell respiration to be removed in the gas exhaust stream, to avoid toxic levels (Gray *et al.*, 1996). The removal of carbon dioxide is known as ventilation.

Different aeration methods have been employed in animal cell culture:

(i) surface aeration;
(ii) aeration through membrane devices or gas-permeable tubing;
(iii) bubble aeration.

The first two methods are widely used just for laboratory-scale applications, since there are limitations for their use at larger scales. In surface aeration, the oxygen transfer rate is related to the liquid surface area (gas–liquid interface). When the culture scale is increased, maintaining constant geometric proportions, the volume increases with the third power of a characteristic linear dimension of the system, whereas the surface area increases with its square.

A recent option is that found in wave bioreactors, where the generation of waves increases oxygen transfer by augmenting both the interfacial area (a) and the global transfer coefficient k_L (Singh, 1999). Therefore, these reactors are already available commercially at volumes up to 500 L.

The second method uses devices that can be positioned either inside the bioreactor or in external recirculation loops. This method allows aeration in the absence of bubbles, minimizing the negative effects that these can exert on the cells, as will be discussed later in this chapter (Lehmann *et al.*, 1987). The aeration devices can be based either on membranes or on non-porous tubes made of oxygen-permeable materials, such as silicon, or on porous devices made of materials that are impermeable to oxygen, such as polypropylene. This method also has limitations regarding scale-up, and its use has been reported just for bioreactors up to 150 L in volume (Lehmann *et al.*, 1987). Its use at a larger scale becomes unfeasible, due to the operational difficulties associated with assembling, cleaning, and sterilizing systems that contain hundreds of meters of tubes or membranes. In heterogeneous bioreactors, external aeration devices are most commonly employed, since it is easy to recirculate a cell-free or almost cell-free stream through an independent oxygenation system. Due to the low solubility of oxygen in aqueous media, the recirculation speed usually needs to be very high, reaching up to 100 vvd (recirculation volume per reactor volume per day).

The aeration method most widely used in animal cell cultures is bubble aeration, just as in microbial fermentations. This method is simple and consists of bubbling a gas stream directly into the culture medium, using a

sinter sparger, orifice sparger or jet-flow device. Sinter spargers are made of porous stainless steel and can generate bubbles with diameters in the order of hundreds of micrometers. Orifice-type and jet-flow spargers generate bubbles 1 mm in diameter or larger (Puleo *et al.*, 2004). In the bioreactors, the spargers are generally positioned below the impellers, to promote a homogeneous distribution of bubbles inside the culture vessel.

Although simple, the use of bubble aeration in animal cell cultivation requires the solution of certain technological challenges for its implementation, since one of the most characteristic features of animal cells is their low resistance to mechanical stresses. Cell membranes have a mechanical resistance that is much lower than that of microbial cell walls. According to Castilho and Anspach (2003), shear stresses in the order of 60 Pa are critical for cell death.

It is reported that bubble disengagement and bursting at the liquid surface is a major cause of cell death in animal cell cultures (Kunas and Papoutsakis, 1990; Butler, 2004). The cell membrane is slightly hydrophobic under culture conditions, and therefore cells tend to adhere to bubbles ascending inside a bioreactor. When these reach the liquid surface, the film of fluid that surrounds the bubble begins to be drained by gravity, decreasing in thickness. The bubble bursts at the moment the mechanical resistance of this film is not sufficient to resist the pressure inside the bubble. The explosion of a bubble causes large velocity gradients in the liquid below the bubble. *Figure 9.18* shows that the presence of a bubble creates a cavity in the liquid and, at the moment of bubble bursting, this cavity is quickly filled with liquid, generating high velocity gradients within short distances, resulting in shear stress levels that are sufficiently high to destroy the cells found in this region (Meier *et al.*, 1999).

In general, to minimize damage to the cells, very small bubbles should be avoided in animal cell cultures, since the interfacial area of the bubbles

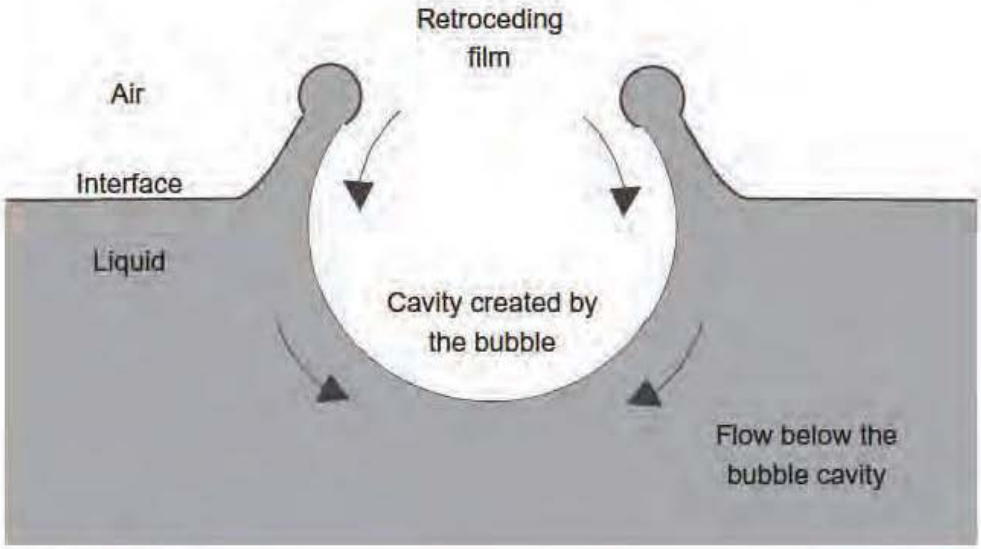


Figure 9.18

Dynamics of bubble explosion (adapted from Wu and Goosen, 1995).

is intrinsically related to the kinetics of cell death in bioreactors (Tramper *et al.*, 1988; Wu and Goosen, 1995). Small bubbles provide a greater total interfacial area than large bubbles. Therefore, small bubbles are able to carry more cells to the top gas–liquid interface, where cell damage occurs due to bubble explosion. On the other hand, small bubbles can transfer oxygen more efficiently, and this allows the use of much lower aeration rates. Thus, all these factors should be evaluated when determining the most adequate operational conditions.

The addition of surfactants allows a modification of the kinetics of non-specific cell adhesion to bubbles. When substances such as methyl cellulose or Pluronic® F68 are added to the culture, the time needed for cell adhesion to occur is increased (Meier *et al.*, 1999). In this way, the number of cells adhered to a bubble at the moment of its explosion is lower by several orders of magnitude. The non-ionic surfactant Pluronic® F68 is so far the best option, since it efficiently protects cells from bubble damage without significantly affecting oxygen transfer. However, its presence may be undesirable for certain stages of protein purification.

Foam formation is another problem that occurs in bubble-aerated bioreactors, especially in the presence of serum-containing culture media or at high protein concentrations (Butler, 2004). An uncontrolled accumulation of foam in the upper part of the equipment can occur, resulting eventually in severe problems due to blockage of exhaust gas filters. As a consequence, gas transfer through the surface is seriously affected. To decrease the negative effects of foam, different approaches can be adopted: (a) chemical antifoams; (b) foam traps; (c) bubble-free aeration; or (d) low aeration rates using pure oxygen.

The use of silicon-based antifoams is common in industry (van Bonarius *et al.*, 1993). However, they should be used with care, since these substances can be toxic to the cell above certain concentrations. Furthermore, chemical antifoams can pose problems for the chromatographic purification of the product. Foam traps, which are devices mounted in the upper part of bioreactors to break the foam, have been used successfully at small and intermediate scales, but are not widely used on a large scale. On the other hand, low aeration rates using pure oxygen effectively lead to a significant decrease or even complete elimination of foam, but may result in CO_2 accumulation in the medium, which is harmful to the cells (Gray *et al.*, 1996).

Both aeration and ventilation are enhanced by mechanical agitation of the cell suspension. However, agitation also has other functions in a bioreactor: to maintain cells in suspension (in the case of homogeneous bioreactors or suspended microcarriers), as well as to homogenize the fluid. This homogenization is important to avoid the appearance of dead zones and of nutrient, metabolite, and temperature gradients, as well as to promote the transfer of heat and of the different chemical species, including oxygen, in order not to limit the performance of the biological system.

When stirred-tank bioreactors started to be used for animal cell cultivation, many problems related to deleterious effects of agitation on cell viability were observed. However, it was noted that the use of large impellers rotating at low speeds could minimize mechanical damage to the cells. The most widely used impeller types are marine (Chisti, 1993)

and inclined-blade impellers (Michaels *et al.*, 1996). On a small scale, stirrers with oscillating membranes (Lehmann *et al.*, 1987) have also been proposed. However, these and other similar systems have limited efficiency and are difficult to scale-up. Currently, marine propellers continue to be the most widely used impeller type, but also radial-flow impellers, such as turbines, or combinations of different types are utilized (Krahe, 2003).

The flow pattern in a stirred bioreactor with baffles is extremely complex and, generally, turbulent under normal operational conditions (Brucato *et al.*, 1998). Higher shear rates and, consequently, higher energy dissipation rates occur near smaller turbulent eddies, that rotate at higher velocities. The smallest size an eddy can reach under given flow conditions is known as microscale of turbulence and can be estimated through Kolmogorov's equation (Joshi *et al.*, 1996):

$$\lambda = \left(\frac{\nu^3}{\varepsilon}\right)^{0.25} \tag{18}$$

Where:

λ = Kolmogorov eddy length (microscale of turbulence);
ν = kinematic viscosity;
ε = energy dissipation per unit mass.

Several authors have proposed that, when the microscale of turbulence is of the same order or smaller than the cell diameter, the energy dissipation due to the eddy action on the cell surface can destroy the cells (Kunas and Papoutsakis, 1990). In the case of larger eddies, cells can be accommodated in their interior, moving at the same velocity as the eddies. Thus, no velocity gradients are formed that could damage the cells. However, other authors state that adopting Kolmogorov's theory to explain cell damage is inadequate, mainly due to the evidence that the dominating deleterious effects on cells are due to bubble explosion at the gas–liquid interface (Kioukia *et al.*, 1992). Furthermore, it has been demonstrated that in the absence of a gas phase inside the bioreactor, cells are more resistant to high rotation speeds than when bubbles are present (Kunas and Papoutsakis, 1990).

9.6 Scale-up

To meet the demand for a product in the market, two approaches are possible; process intensification by increasing process productivity, or increasing the production scale. The latter approach may be achieved by increasing the number of bioreactors of a given size or by using bioreactors of larger volume. From the economic viewpoint, generally the second option is more adequate, since equipment costs usually increase with scale to the power of 0.6 (Rouf *et al.*, 2000). Beyond that, operation and maintenance costs increase with the number of pieces of equipment, indicating that for scale-up an increase in the bioreactor volume is more advantageous than using several smaller units.

The aim of scaling-up is to reproduce in production scale the conditions optimized at laboratory and/or pilot scale. Most of the general principles

adopted for the scale-up of microbial fermentation processes are applicable to animal cell culture. Usually the factors that can influence volumetric productivity are investigated when changing the scale in order to identify those that are most critical. Once they are identified, similar criteria for these factors are established for the different scales. The most commonly used criteria concern the peripheral rotation velocity of the impeller and the gas flow rate, in the case of bubble-aeration processes.

Before scaling-up a process established at a small scale, it is important that it is appropriately characterized and optimized. First, a cell line with desirable production, growth, and genetic stability should be selected. This selection is usually carried out in multiwell plates and stationary flasks (Castillo *et al.*, 2005). An adaptation to suspension growth is usually desirable. Then, the master and working cell banks are prepared, to cryopreserve the selected cell line. Subsequently, process development is initiated. Studies on the behavior of the cell line are carried out in laboratory bioreactors that should have the same configuration as those to be used at a production scale. Under these conditions, the kinetic characteristics, production pattern, oxygen requirements, and shear resistance of the cell line are determined (Víctores, 2005). After that, a mode of operation should be chosen and the operational variables that maximize volumetric productivity should be determined. Finally, studies aimed at process scale-up should then be carried out.

The stirred-tank bioreactor is an example of a reactor that is scaled-up by direct increase of its volume. One of the critical parameters that should be evaluated at different scales is the rotation speed of the impeller. Different criteria can be employed:

(i) to maintain constant the power per fluid volume dissipated by the impeller;
(ii) to keep constant the peripheral velocity of the impeller;
(iii) to maintain constant the volumetric oxygen transfer coefficient (k_La).

Each of these criteria will give different results, so that they should be carefully evaluated, preferably from experimental results. For the aeration parameters, it is important to evaluate which is the most relevant phenomenon; the capacity of bubbles for oxygen transfer or their negative effect on the cells (Chisti, 2000). At very large scales, the ventilation is also a very important issue, mainly if low aeration rates with pure oxygen are used (Gray *et al.*, 1996).

Since in animal cell culture processes the effects of mechanical stress are much more relevant than in microbial fermentations (Chisti, 1993), it is quite common to adopt scale-up criteria that are associated with cell damage (Joshi *et al.*, 1996), such as constant peripheral impeller velocity, constant aeration rate, and constant integrated shear stress (Croughan *et al.*, 1987).

When bioreactors coupled to cell retention devices are used, it is also necessary to evaluate the scale-up of the cell separation equipment. In the case of the spin-filter (see Chapter 11), parameters such as filter rotation velocity and the ratio of filtration area to bioreactor working volume are particularly relevant (Deo *et al.*, 1996).

9.7 Economic aspects relevant to bioreactor selection: the productivity factor

Most proteins produced by animal cell culture on a large scale are innovative biopharmaceuticals, protected by patents, that have a selling price several times higher than the production costs. Those prices are justified by the large investments in research and development for developing a new biopharmaceutical, which are calculated to be in the range of 2 to 250 million US dollars (Savage, 2000).

The complex production facilities required for the cultivation of animal cells, with sophisticated auxiliary services that should avoid contamination and toxicity in the cultures, and fitted with highly automated and validatable equipment, are the reasons why the main cost component of these processes is associated with equipment and other technical components (Petrides, 2000).

That is why the main economic consequence of animal cell process optimization is the decrease of the scale of equipment needed to meet a given product demand. This can reduce the investment required to build a production facility, which varies in the range of 10 to 300 million US dollars (Petrides, 2000).

Volumetric productivity of a process is a parameter that determines the size of the facilities needed to obtain a given annual production, thus determining the economic efficiency of protein production based on animal cells. Volumetric productivity, as well as the annual production, can be calculated from Equations 19 to 22:

$$P_{acc} = V q_p \int X_v \, dt \tag{19}$$

$$t_{proc} = t_{prep} + t_{growth} + t_{prod} \tag{20}$$

$$P_V = \frac{P_{acc}}{t_{proc} \; V} \tag{21}$$

$$P_A = P_V V t_{work} \tag{22}$$

Where:

P_{acc} = amount of product accumulated throughout a run;

t_{proc} = process time;

t_{prep} = time required for the preparation of the bioreactor (includes cleaning and sterilization);

t_{growth} = time needed for cells to grow up to the concentration desired for the process. This time is a function of the specific growth rate of the cell line and of the inoculation density;

t_{prod} = duration of the production phase. In the case of cell lines that do not require induction to express the protein product, this time period also includes the cell growth phase;

P_V = volumetric productivity of the bioreactor;

P_A = annual production obtained in the bioreactor;

t_{work} = number of working days per year (generally 300 days or 44 weeks per year).

It is important to emphasize the general character of this definition for the volumetric productivity (P_V), since it includes all phases of a production cycle in a bioreactor, allowing an evaluation of the impact of bioreactor preparation time and duration of growth and production phases on productivity. As can be observed from Equations 19 to 22, for an industrial bioreactor with a given volume and operation mode, the volumetric productivity depends basically on cell concentration in the production phase and on the specific product formation rate (q_P).

The values of q_P can vary significantly as a function of the expression system used and of the recombinant protein product. In general, values in the range of 1–30 pg cell ^{1} day ^{1} are reported for stably transfected cell lines. However, Chico and Jäger (2000) have reported values as high as 85 pg cell ^{1} day ^{1} for the expression of a protein using the insect cell-baculovirus expression vector system. This value approaches the maximum theoretical value that is expected for animal cells, which is 100 pg cell ^{1} day ^{1} (Ozturk, 1990).

In this way, the use of bioreactors that allow high cell density cultures is of great importance as a tool to increase process productivity. *Table 9.3* shows volumetric productivity values that can be obtained in the main types of industrial bioreactors, as well as the typical working volume and the cell concentration ranges that are usually attained.

As can be observed in *Table 9.3*, cell concentrations equal to or higher than 10^7 cells ml ^{1} are needed to attain volumetric productivities higher than 50 mg L ^{1} d ^{1}. Also, it is important to note that as cell concentration increases, the complexity of the process increases and, consequently, bioreactor scale-up becomes limited, for example by physical limitations of materials used in hollow-fiber cartridges.

Figure 9.19 shows typical cell concentrations reached in the main industrial bioreactors and a comparison of these values with those found in microbial fermentations. As can be observed, batch and fed-batch cultivations attain dry biomass values comparable to those of continuous cultures of microorganisms, so that mass and heat transfer capacities are not limited for these operation modes. However, high cell density cultivation in heterogeneous bioreactors, such as hollow-fiber devices, reaches dry biomass values similar to the maxima observed in microbial cultures.

Table 9.3 Comparison of working parameters and typical productivities found for the main industrial bioreactors

Parameter	Stirred tank bioreactors operated in batch and fed batch mode	Stirred tank bioreactors operated in perfusion mode	Heterogeneous bioreactors (packed bed or hollow fiber) operated in perfusion mode
Cell concentration (cells mL^{-1})	10^6	10^7	10^8 10^9
Complexity	Low	Medium	High
Bioreactor volume*	Up to 20 000 L	100 2000 L	0.2 10 L
Productivity (mg L^{-1} day^{-1})	10 40	50 600	500 1000

*Considering only the volume of the bioreactor compartment where cells grow.

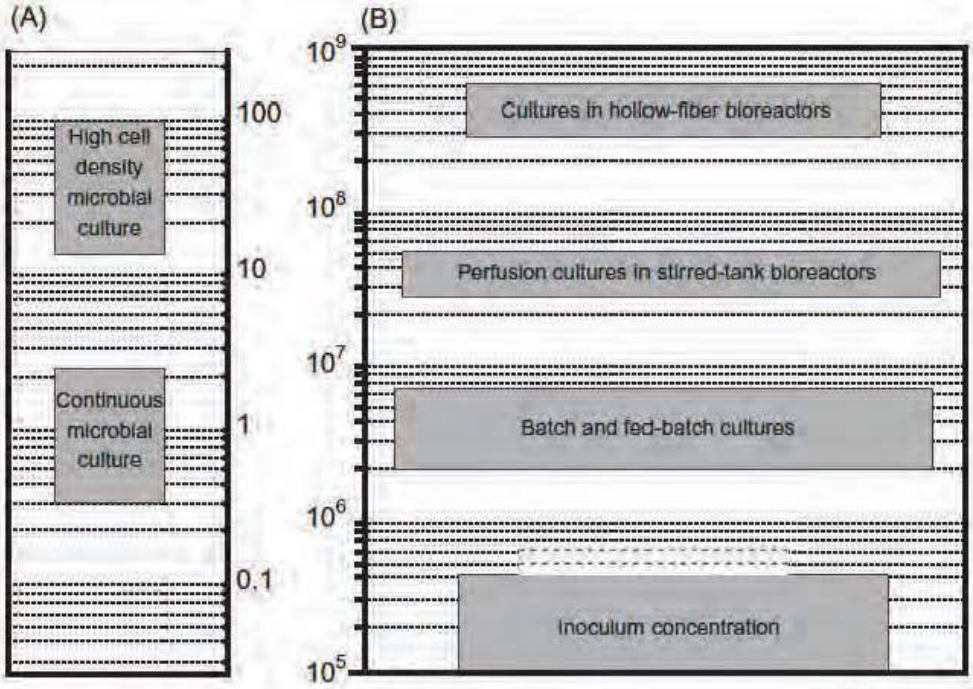


Figure 9.19

Ranges of cell concentration reached in: (A) microbial bioprocesses (concentration expressed as gL^{-1} of dry biomass); (B) animal cell cultures (concentration expressed as cells mL^{-1}), considering 10^6 cells = 0.25 mg of dry biomass (adapted from Frame and Hu, 1990).

Figure 9.19 also suggests that there is an optimization window available; developing new cell culture methods that provide cell concentrations in the range of 10^7 to 10^8 cells mL^{-1} could represent requirements still lower than in microbial fermentations, but at the same time a significant increase in the volumetric productivities of industrial animal cell processes.

As evidenced by Equation 22, the volumetric productivity (P_V) determines the cultivation volume (V) needed to reach a desired annual production (P_A). Application of this equation for volumetric productivities up to 500 mg L^{-1} d^{-1} is illustrated in *Figure 9.20* and allows an estimate of the total culture volume required for obtaining production capacities in the range of 1–1000 kg per year.

As a result of recent advances in the use of recombinant antibodies for the treatment of chronic diseases and several types of cancer, a growing number of new therapeutic antibodies is currently under development (see Chapters 16 and 17). As a consequence, large investments in the construction of industrial animal cell facilities are being made, surpassing 1 million liters in terms of total installed capacity. In the recent years, biotechnology companies such as Genentech Inc. in the USA and Boehringer Ingelheim in Germany constructed plants with a total bioreactor volume of more than 100 000 L and production capacity higher than 1000 kg of antibodies per year, after investments in the order of hundreds of million US dollars.

However, from the relationship between culture volume and production capacity reported by many companies, it is possible to observe that volumetric productivities lower than 50 mg L^{-1} d^{-1} are still quite usual.

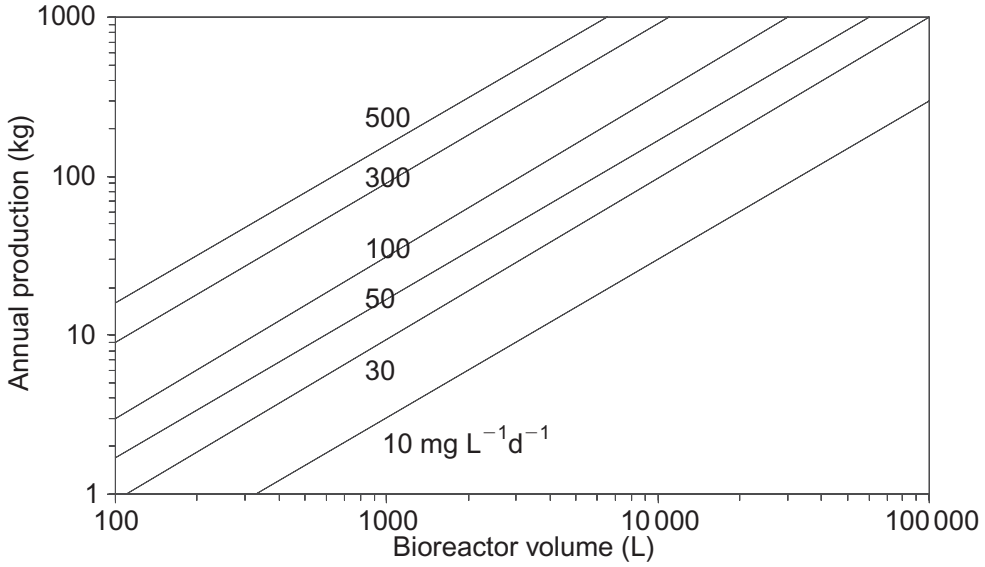


Figure 9.20

Relationship between annual production and culture volume, for different levels of volumetric productivity.

Thus, it becomes evident that increases in the volumetric productivity up to levels in the range of 500 mg L ^{1} d ^{1} could lead to a decrease of one order of magnitude in the culture volume needed to obtain a large amount of therapeutic proteins on an industrial scale. Therefore, the use of high productivity bioreactors could represent savings in the range of tens of millions of US dollars in the cost of recombinant protein production facilities, making this technology feasible for smaller companies and for developing countries (Lage, 1994).

References

Altamirano C, Illanes A, Casablancas A, Gámez X, Cairó JJ, Gòdia F (2001), Analysis of CHO cells metabolic redistribution in a glutamate based defined medium in continuous culture, Biotechnol. Prog. 17:1032 1041.

Altamirano C, Paredes C, Illanes A, Cairó JJ, Gòdia F (2004), Strategies for fed batch cultivation of t PA producing CHO cells: substitution of glucose and glutamine and rational design of culture medium, J. Biotechnol. 110:171 179.

Bebbington CR, Renner G, Thomson S, King D, Abrams D, Yarranton GT (1992), High level expression of a recombinant antibody from myeloma cells using a glutamine synthetase gene as an amplifiable selectable marker, Bio/Technol. 10:169 175.

Bibila T, Robinson DK (1995), In pursuit of optimal fed batch process for monoclonal antibody production, Biotechnol. Progr. 11:1 13.

Birch JR, Racher AJ (2006), Antibody production, Adv. Drug Deliv. Rev. 58:671 685.

Bödeker BDG (1994), Production of recombinant factor VIII from perfusion cultures: I Large scale fermentation, In: Spier RE, Griffiths JB, Berthold W (Eds), Animal Cell Technology: Products of Today, Prospects of Tomorrow, Butterworth Heinemann, London, pp. 580 583.

Brucato A, Ciofalo M, Grisafi F, Micale G (1998), Numerical prediction of flow fields

in baffled stirred vessels: a comparison of alternative modelling approaches, Chem. Eng. Sci. 53:3653 3684.
Butler M (2004), Animal Cell Culture and Technology, BIOS Scientific Publishers, London.
Castilho LR, Anspach FB (2003), CFD aided design of a dynamic filter for mammalian cell, Biotechnol. Bioeng. 83:514 524.
Castilho LR, Medronho RA (2002), Cell retention devices for suspended cell perfusion cultures, Adv. Biochem. Eng. Biotechnol. 74:129 169.
Castillo A, Víctores S, Faifer E, Rabasa Y (2005), Development of an integrated strategy for recombinant cell line selection, In: Gòdia F, Fussenegger M (Eds), Animal Cell Technology Meets Genomics, Proceedings of the 18th ESACT Meeting, Springer, Dordrecht, pp. 505 508.
Chico E, Jäger V (2000), Perfusion culture of baculovirus infected BTI Tn 5B1 4 insect cells: a method to restore cell specific β trace glycoprotein productivity at high cell density, Biotechnol. Bioeng. 70:574 586.
Chisti Y (1993), Animal cell culture in stirred bioreactors: observations on scale up, Process Biochem. 28:511 517.
Chisti Y (2000), Animal cell damage in sparged bioreactors, Trends Biotechnol. 18:420 432.
Chu L, Robinson DK (2001), Industrial choices for protein production by large scale cell culture, Curr. Opin. Biotechnol. 12:180 187.
Corning (2006), available at: http://www.corning.com (accessed November 2006).
Croughan MS, Hamel JF, Wang DI (1987), Hydrodynamic effects on animal cells grown in microcarrier cultures, Biotechnol. Bioeng. 29:130 141.
Deo YM, Mahadevan MD, Fuchs R (1996), Practical considerations in operation and scale up of spin filter based bioreactors for monoclonal antibody production, Biotechnol. Progr. 12:57 64.
Fassnacht D, Porter R (1999), Experimental and theoretical considerations on the oxygen supply of animal fixed bed culture, J. Biotechnol. 72:169 184.
Figueredo A (2002), Evaluación de la Tecnología de Perfusión en Bioreactores de Tanque Agitado como Alternativa Productiva para la Obtención del Anticuerpo hR3, M.Sc. Thesis, ISPJAE, Cuba.
Frame KK, Hu WS (1990), Cell volume measurement as an estimation of mammalian cell biomass, Biotechnol. Bioeng. 36:191 197.
GE Healthcare (2006), Microcarrier Cell Culture: Principles and Methods Handbook. Available at: http://www5.amershambiosciences.com/aptrix/upp00919.nsf/Content/WD:Microcarrier+ce(148896581 B345) (accessed November 2006).
Goochee CF, Monica T (1990), Environmental effects on protein glycosylation, BioTechnol. (NY) 8:421 427.
Gramer MJ, Poeschl DM, Conroy MJ, Hammer BE (1999), Effect of harvesting protocol on performance of a hollow fiber bioreactor, Biotechnol. Bioeng. 65:334 340.
Gray DR, Chen S, Howarth W, Inlow D and Maiorella BL (1996), CO_2 in large scale and high density CHO cell perfusion culture, Cytotechnology 22:65 78.
Hassell T, Gleave S, Butler M (1991), Growth inhibition in animal cell culture: the effect of lactate and ammonia, Appl. Biochem. Biotechnol. 1:29 41.
Himmelfarb P, Thayer PS, Martin HE (1969), Spin filter culture: the propagation of mammalian cells in suspension, Science 164:555 557.
Jo EC, Yun JW, Jung KH, Chung SI, Kim JH (1998), Performance study of perfusion cultures for the production of single chain urokinase type plasminogen activator (scu PA) in a 2.5 L spin filter bioreactor, Bioproc. Eng. 19:363 372.
Joshi JB, Elias CB, Patole MS (1996), Role of hydrodynamic shear in the cultivation of animal, plant and microbial cells, Chem. Eng. J. 62:121 141.
Kadouri A, Spier RE (1997), Some myths and messages concerning the batch and

continuous culture of animal cells, Cytotechnology 24:89 98.
Kempken R, Büntemeyer H, Lehmann J (1991), The medium cycle bioreactor (MCB): monoclonal antibody production in a new economic production system, Cyto technology 1:63 74.
Kioukia N, Nienow AW, Emery AN, Al Rubeai M (1992), The impact of fluid dynamics on the biological performance of free suspension animal cell culture: further studies, Trans. I. Chem. Eng. 70:143 148.
Krahe M (2003), Biochemical Engineering, In: Ullmann's Encyclopedia of Industrial Chemistry, Wiley VCH, Weinheim.
Kunas KT, Papoutsakis ET (1990), Damage mechanisms of suspended animal cells in agitated bioreactors with and without bubble entrainment, Biotechnol. Bioeng. 36:476 483.
Lage A (1994), Biotechnology: offering new opportunities for developing countries, Biopharm Eugene 75:18 20.
Lehmann J, Piehl GW, Schulz R (1987), Bubble free cell culture aeration with porous moving membranes, Dev. Biol. Stand. 66:227 240.
Lonza (2006), available at: http://www.lonza.com/group/en/products services/ custommanufactoring/mammalian/manu capabilities.html (accessed November 2006).
Meier SJ, Hatton TA, Wang DIC (1999), Cell death from bursting bubbles: role of cell attachment to rising bubbles in sparged reactors, Biotechnol. Bioeng. 62:468 478.
Michaels JD, Mallik AK, Papoutsakis ET (1996), Sparging and agitation induced injury of cultured animal cells do cell to bubble interactions in the bulk liquid injure cells?, Biotechnol. Bioeng. 51:399 409.
Morbach S, Krämer R (2003), Impact of transport processes in the osmotic response of *Corynebacterium glutamicum*, J. Biotechnol. 104:69 75.
Nunc (2006), Available at: http:»www.nuncbrand.com (accessed November 2006).
Ozturk SS (1990), Kinetics and Metabolism of Antibody Production by Hybridoma Cells, Doctoral Thesis, Michigan University, USA.
Ozturk SS (1996), Engineering challenges in high density cell culture systems, Cyto technology 22:3 16.
Petrides D (2000), Bioprocess Design, Bioseparation Textbook (Intelligent Inc., Super pro Designer), available at: http://www.intelligen.com (accessed November 2006).
Puleo JA, Johnson RV, Kooney TN (2004), Laboratory air bubble generation of various size distributions, Rev. Sci. Instr. 75:4558 4563.
Robinson DK (1994), Characterization of a recombinant Ab produced in the course of a high yield feedbatch process, Biotechnol. Bioeng. 44:727 733.
Rodríguez G, Arias MA, Suarez J, Chea M, Bouzó L, Cuervo R, Alvarez I, Chico E (2005), Optimizing cultivation strategies in different scales of hollow fiber bioreactors, In: Gòdia F, Fussenegger M (Eds), Animal Cell Technology Meets Genomics, Proceedings of the 18th ESACT Meeting, Springer, Dordrecht, pp. 747 750.
Rouf SA, Moo Young M, Scharer JM, Douglas PL (2000), Single versus multiple bioreactor scale up: economy for high value products, Biochem. Eng. J. 6:25 31.
Rupp R (1987), Cellular microencapsulation for large scale production of monoclonal antibodies, In: Tolbert WR, Feder J (Eds), Large Scale Cell Culture Technology, BK Lyndersen Hanser Publisher, Munich.
Savage C (2000), Scale up and bioproduction, Genet. Eng. News, 20:1 69.
Singh V (1999), Disposable bioreactor for cell culture using wave induced agitation, Cytotechnology 30:149 158.
Tramper J, Smit D, Straatman J, Vlak JM (1988), Bubble column design for growth of fragile insect cells, Bioproc. Biosyst. Eng. 3:37 41.
van Bonarius D, van de Wouw G, Tramper J (1993), Effect of silicone antifoam on shear sensitivity of hybridoma cells in sparged cultures, Biotechnol. Progr. 9:

504 509.
van Wezel AL (1967), Growth of cell strains and primary cells on micro carriers in homogeneous culture, Nature 216:64 65.
Velez D, Reuveny S, Miller L, MacMillan JD (1987), Effect of feeding rate on monoclonal antibody production in a modified perfusion fed fermentor, J. Im munol. Methods 102:275 278.
Víctores S (2005), Selección y Caracterización de Líneas de Mieloma NS0 Recombi nante con el Fin de Escalado Productivo, Final year Project in Chemical Engineering, ISPJAE, Cuba.
Vits H, Hu WS (1992), Fluctuations in continuous mammalian cell bioreactors with retention, Biotechnol. Progr. 8:397 403.
Wang MD, Yang M, Huzel N, Butler M (2002), Erythropoietin production from CHO cells grown by continuous culture in a fluidized bed bioreactor, Biotechnol. Bioeng. 77:194 203.
Wang Y, Ouyang F (1999), Bead to bead transfer of Vero cells in microcarrier culture, Cytotechnology 31:221 224.
Wave Biotech (2006), Available at: http://www.wavebiotech.com (accessed November 2006).
Weikert S, Papac D, Briggs J, Cowfer D, Tom S, Gawlitzek M, Lofgren J, Mehta S, Chisholm V, Modi N, Eppler S, Carroll K, Chamow S, Peers D, Berman P, Krummen L (1999), Engineering Chinese hamster ovary cells to maximize sialic acid content of recombinant glycoproteins, Nat. Biotechnol. 17:1116 1121.
Wu J, Goosen MFA (1995), Evaluation of the killing volume of gas bubbles in sparged animal cell culture bioreactors, Enzyme Microb. Technol. 17:1036 1042.
Xie L, Wang DIC (1997), Integrated approaches to the design of media and feeding strategies for fed batch cultures of animal cells, Tibtech 15:109 113.

Monitoring and control of cell cultures

10

Aldo Tonso

10.1 Introduction

Animal cell cultures can be considered, in many ways, similar to cultures of microorganisms, such as bacteria, yeasts, and filamentous molds, presenting several common characteristics, for instance the major variables to be monitored and controlled. However, there are some specific culture variables that may require different strategies. This chapter presents the basic concepts of bioprocess monitoring and control, with emphasis on the specific requirements of the cultures of animal cells. This will start with a description of the general goals of process monitoring and control, a description of the main variables involved, and finally, the most commonly used control strategies.

It should be noted that developments in electronics and computer science have contributed significantly to the improvement of monitoring culture systems and process control. However, there are still some areas for further general improvement of cell culture bioprocess monitoring and control, such as the estimation of cell concentration in real time. Several basic texts may supplement this chapter: Kilburn (1991), Schügerl (1991, 2001), and Sonnleitner (2000).

10.2 Monitoring and control: basic concepts

Any process, industrial or laboratory-based, presents a series of important variables that represent its state. In the case of cell culture, there are the variables related to the environment to which the cells are exposed, such as temperature, pH, dissolved oxygen, nutrients in the culture medium, and metabolite concentrations, as well as those related to the cell itself, such as concentration, average size, or the profile of intracellular enzyme activities. In order to make meaningful decisions during a process it is desirable to have at hand the greatest amount of information available about these variables. At this point, it is important to define some concepts relative to process monitoring and control.

The first relates to the time the information is available. As an example, suppose we know that the glutamine concentration at a certain point of the culture was 0.23 g/L, obtained through a chromatographic run 1 week after the end of the culture. Such information is considered offline and can be used if we study the influence of that substrate in cell growth or

product synthesis. However, if such information were available online, it could be used in an automated system that could replace the nutrient loss by medium replenishment in a fed-batch process. This would ensure that glutamine would not be depleted from the medium. In this case, we can say that the variable glutamine concentration is being monitored in "real time" (also called "online").

Another important concept is whether the acquired information comes from the process place itself, or from a sample that is removed and then analyzed. For instance, in the study of the degree of cell aggregation, if a sample is removed from the culture, it goes through several manipulations before its analysis (such as dilution) and it becomes possible that the observed aggregation does not match that present in the culture. In such cases, the ideal would be to observe the cells through a microscope inserted inside the bioreactor. This would be called *in situ*, or "in its own place".

There are two basic reasons to have process information by monitoring:

(i) Better knowledge of the process itself, as part of research or in the search for cause and effect. For instance, when the pH is below a certain value, the growth of cells decreases. This knowledge is also fundamental in the development of a model that would represent the process under study (see Chapter 8).
(ii) Use of certain information, in real time, to take some control action. For instance, based on the dissolved oxygen concentration at a certain moment, it is possible to act on the aeration flow, to avoid limitations to cellular metabolism.

Control refers to the actions taken in the process. A flow chart of a typical control loop is shown, using the temperature control of a bioreactor as an example.

As can be observed in *Figure 10.1*, information from the process (value of the temperature measured by the sensor) arrives at the control loop, which defines it as a "closed loop." This is the case for most controllers installed in bioreactors. If the decisions of a controller do not take into account any of the monitored information of the process, it is called an "open loop." An example of an open loop is a fed-batch process, where it

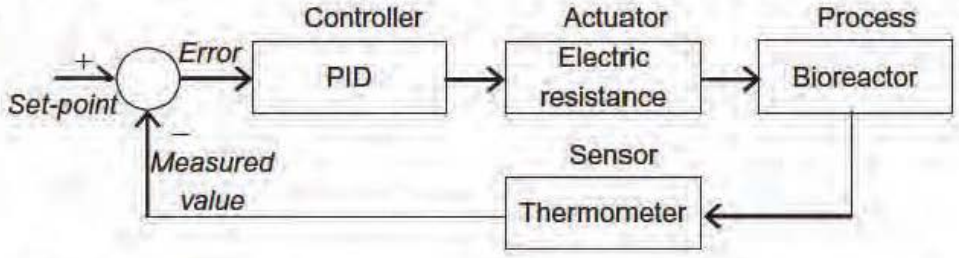


Figure 10.1

Flow chart of a typical control loop showing temperature control elements: a desired temperature value (set point) is compared to the measured value by the thermometer (sensor) and, based on the error measurement, a signal to the electric resistance (actuator) is generated by the controller, that will heat up the bioreactor (process).

is desired to keep a nutrient concentration at a certain level, but the flow rate of the nutrient feed is fixed beforehand (*a priori*). As there is no information from the process, that control system is not able to answer to the behavior fluctuations of the cells during growth.

10.3 Particular characteristics of cell cultures

Animal cell cultures, either from mammalian or insect cells, show specific characteristics that differ from microbial cultures (bacteria, yeasts, and filamentous molds). Among the characteristics that most interfere with monitoring methodologies and process control (and that will be approached with more detail in the present chapter), are the following.

(i) Slow growth, with specific rates around 10–20 times lower than microbial cells. This has encouraged the development of bioreactors with cell retention devices (see Chapter 11), where real-time estimations of cell concentration or nutrients are often necessary. Longer cultures (of the order of several days) require more robust sensors (such as pH and dissolved oxygen) or the possibility of replacing electrodes during the process. Also, any contamination has a greater effect on the process, demanding more advanced sterility maintenance systems.
(ii) Requirement of a solid support (substrata) for cells, in the case of anchorage-dependent cell lines. This causes problems for the estimation of cell concentration.
(iii) Mechanical fragility because of the absence of a cell wall, which is present in most microbial cells. Animal cells are adapted to grow in tissue structures, which provide mechanical support *in vivo* and which are lost in culture. As a consequence of the fragility, alternative methods of oxygen transfer have been developed, such as bubble-free systems (see Chapter 9). Such systems require a suitable strategy for monitoring and control of the variables involved in oxygen transfer.
(iv) The commonly used method of pH buffering by the manipulation of the bicarbonate/carbon dioxide concentrations. This alters the overall gas system control.

10.4 Main bioprocess variables

10.4.1 Temperature

Whatever the scale or method of culture (T-flask, Schott bottle, spinner flask, or bioreactor), the temperature of the culture medium with which the cells are in contact is always a fundamental state variable, because it interferes with growth and the production process. However, it is a process variable that is easy to monitor and control. On a small scale the culture flask is usually put in a thermostatically controlled incubator, where the measured value of a thermometer sends a sign to turn the heating on or off (“on–off control”). In bioreactors, there are equivalent systems, as will be seen later. Usually, however, a resistance thermometer sensor type is used (resistance temperature detector or RTD), the electric

resistance of which varies according to the temperature of its platinum sensor (Pt100), being typically 100 Ω at 0°C and 123 Ω at 60°C.

The most common temperature control systems for cell culture are the following:

(i) Heat blanket around the bioreactor, the electric resistance of which can be turned on or off as required to heat the system. In this case, the minimum culture temperature is limited by the room temperature, which can be a problem in cultures of insect cells (with optima around 28°C) in facilities without air conditioning.
(ii) Water-jacket reactor with a temperature sensor, which sends a signal to the controller that triggers an electrical heating element within the water of the jacket (to increase the temperature) or open a solenoid valve that allows the inlet of colder water to the circuit (to decrease the temperature). In this case, the minimum temperature of culture is limited by the temperature of the water supply. If the water temperature supply is not sufficiently low, a thermostatically refrigerated bath system can be used.
(iii) Water-jacket reactor with two temperature sensors, for the jacket and for the culture medium. This is different from the control system in (ii) in that the control is made in a cascade, where the medium temperature controller (master) acts to send a set-point value to the jacket water temperature controller (slave). See Section 10.5.1(iii).

Typically, the process is set to control temperature to a value that results in high cell growth or production rate. Sometimes, however, a different temperature may be selected, which could result in a better quality product or a respiration rate compatible with the oxygen transfer system (Chuppa *et al*., 1996). For some processes, a temperature shift batch culture may be desirable in which there are two controlled temperature values, one for the growth phase and another for the production phase, in which the cell division is interrupted due to the low temperature (Fox *et al*., 2004).

10.4.2 pH

pH control systems in cell culture can be classified into four basic types, as follows.

(i) Culture medium buffered naturally by the dissolved solutes. This may allow some pH variation during the culture and in which usually pH electrodes are not used.
(ii) Culture medium buffered with sodium bicarbonate and carbon dioxide gas. In this case, the flask is maintained in an incubator with carbon dioxide gas control, and the concentration of H^+. The pH will remain relatively constant in an acceptable range, due to the equilibrium:

$$CO_2 + H_2O \leftrightarrow H_2CO_3 \leftrightarrow H^+ + HCO_3$$

(iii) Controlled addition of CO_2 in the gas sparged into the bioreactor (see Section 10.4.5), based on the signal of a pH electrode inserted in the culture medium.
(iv) Controlled addition of an alkali solution (such as sodium hydroxide) and/or acid (such as hydrochloric acid) to the bioreactor vessel, based on the signal of a pH electrode.

The most widely used pH electrode in cell culture is the silver/silver chloride electrochemical type with internal gel (that can be sterilized when the whole bioreactor is autoclaved), or the combined type (which can be pressurized during sterilization, to compensate the medium pressurization and to avoid contamination of the sensor with culture medium) (Schügerl, 1991).

In long duration cultures (several weeks), a retractable housing for pH and dissolved oxygen electrodes can be used. With the use of an internal chamber, the electrode can be removed from contact with the culture medium and then recalibrated, repaired, or replaced. After re-installation in the chamber, it is sterilized with direct steam and afterwards re-enters in contact with the culture medium, without risk of contaminations (Sonnleitner, 2000).

There has also been an increase in the ability to measure the intracellular pH, by flow cytometry with pH-dependent fluorescence indicators (Ozkan and Mutharasan, 2002) and nuclear magnetic resonance. There are several reports that relate the intracellular pH value to cell behavior, including metabolism, apoptosis, and specific growth rate (Cherlet and Marc, 1998). Such offline measurements help to interpret the physiological state of the cells, but still cannot be used as control systems.

10.4.3 Dissolved oxygen

Probably the most important state variable for the process control is the dissolved oxygen concentration (DO) in contact with the cells. Because of the very low solubility of oxygen in water and in culture medium, around 6–8 mg/L at cell culture temperature, it is clear that DO is a nutrient that needs to be supplied continuously to the culture medium, and that will be consumed simultaneously with the carbon source in aerobic cultures (Henzler and Kauling, 1993; Moore *et al.,* 1995).

There are several systems of oxygen transfer, but some are specifically used in cell culture in agitated tanks: diffusion through liquid surface, direct sparging, microsparging, and diffusion through membranes. Although the specific respiration rate of animal cells is considerably lower than with microbial cells, it is important to supply oxygen with minimal potential shear stress to the cells caused by agitation and by the rupture of the gas bubbles.

Nowadays, the most commonly used DO electrode is the polarographic that employs an electrochemical method, through the reduction of molecular oxygen at the platinum electrode (cathode) generating electrons for the silver oxidation at the silver/silver chloride electrode (anode). In this type of sensor, it is necessary to impose an external voltage and the resulting current is measured and converted into dissolved oxygen concen-

tration (*Figure 10.2*). Another common type is the galvanic sensor, in which the oxidation reaction at the anode is capable of generating a voltage spontaneously ($Pb \rightarrow Pb^{+2} + 2\ e$) (Johnson *et al*., 2000).

More recently, other electrodes have been developed, the most notable among them are those that use the fluorescence quenching by oxygen on ruthenium compounds (O'Neal *et al*., 2004).

Dissolved oxygen control strategies are associated with the oxygen transfer term in the oxygen mass balance (Equation 1):

$$\frac{dC}{dt} = k_L a.(C_S - C) - Q_{O2}.X \quad (1)$$

where C is dissolved oxygen concentration in culture medium, t is time, k_La is volumetric coefficient of oxygen transfer, C_S is oxygen concentration in balance with the gas (saturation), Q_{O2} is specific respiration rate and X is cell concentration (a good explanation of that equation can be obtained from Bailey and Ollis, 1986).

Typically, oxygen transfer can be modified acting through the rate of impeller agitation or through the aeration or micro-aeration flow rate, which change the k_La term of Equation 1. The dissolved oxygen controller

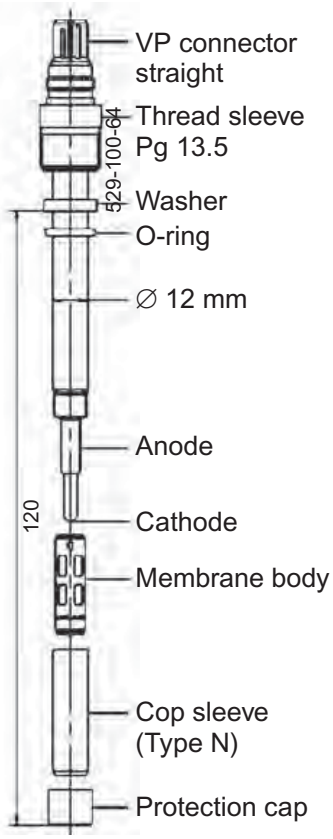


Figure 10.2

Dissolved oxygen polarographic electrode (InPro® 6050, Mettler Toledo, 2006). The cathode, where the half reaction with O_2 ($O_2 + 2H_2O + 4e^- \rightarrow 4OH^-$) takes place, is in contact with a membrane, that allows the transport of the dissolved oxygen from the external medium. At the anode, the silver oxidation ($Ag + Cl^- \rightarrow AgCl + e^-$) takes place.

can calculate new values for a set-point based on the agitation motor speed or the flow rate controller. A disadvantage of this control method is that it interferes with the shear stress imposed by higher values of agitation or aeration, which can be particularly critical in animal cell cultures.

To avoid the above problem a system involving the control of a mixture of two gases can be used, for instance, oxygen and nitrogen (*Figure 10.3*). The dissolved oxygen concentration value is controlled by acting on the two gas flow rates ratio, which varies the C_S value and the oxygen transfer. In this way, it is possible to keep the agitation and the total gas flow rate admitted into the reactor constant and, consequently, the k_La and the stress induced to the cell are also constant.

Alternatively, a membrane of material allowing oxygen diffusion can be used (usually silicone tubing, a microporous material, or polypropylene), avoiding bubble formation. If aeration is accomplished by a bubble-free system (see Chapter 9), using membranes or tubings made of materials that allow oxygen diffusion (e.g. silicone tubing or microporous membranes made of polypropylene), bubble formation is avoided. In this situation, the transfer is not regulated by gas flow or agitation, but instead by the partial pressure of oxygen, which is related to the internal pressure in the membrane and its gas composition and will determine the C_S value (Qi *et al.*, 2003).

An additional advantage of this DO control system is the possibility of having a real-time estimation of the cells' respiration rate. As can be observed in Equation 1, if C is constant, the term of the transfer $k_La.(C_S-C)$ is equivalent to the consumption $Q_{O2}.X$. With the actuation value of the controller, the transfer rate can be calculated, and also the consumption at each moment (Kamen *et al.*, 1996). There are several reports in the literature dealing with the use of respiration measurements to estimate cell concentration online, based on the assumption that, during the growth phase, each cell consumes a constant amount of oxygen (Yoon and Konstantinov, 1994; Ruffieux *et al.*, 1998; Jorjani and Ozturk, 1999).

10.4.4 Cell concentration

In every bioprocess, monitoring biomass concentration is fundamental, because usually the growth is required to be maximized. Substrate con-

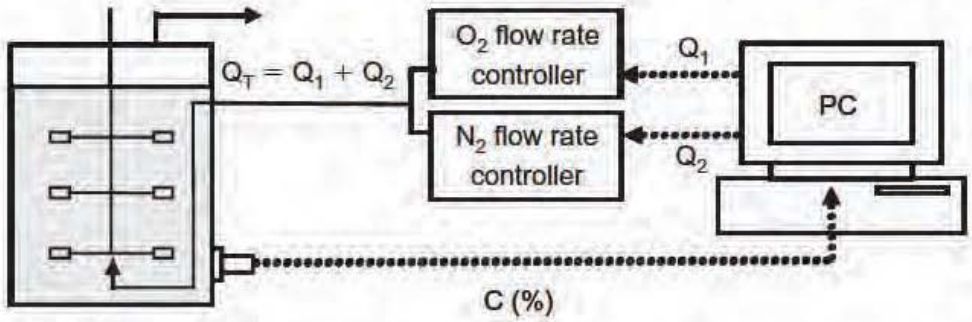


Figure 10.3

Dissolved oxygen control by the adjustment of the oxygen fraction in the sparged gas. Its total flow rate is kept constant and corresponds to the sum of the two controlled gases Q1 and Q2.

sumption or product synthesis rates can be analyzed but it is important that variations in these parameters can be related to the cell concentration. For this reason specific rates are often used, such as the specific rate of product formation:

$$\mu_P = \frac{1}{X} \cdot \frac{dP}{dt} \tag{2}$$

where μ_P is the specific rate of product formation, t is time, X is cell concentration, and P is product concentration.

For microbial cultures it is common to express cell concentration as dry cell weight by volume, in animal cell cultures the number of cells per unit volume is more commonly used (for instance, 1.3×10^6 cells/ml). For the determination of cell number a microscope graduated slide can be used such as a hemocytometer or Neubauer chamber. In these, the thickness between the slide and the coverslip is fixed and known (typically 0.1 mm) and graduation marks on the slide allow the number of cells to be counted in a certain area. The volume can be determined by multiplying this area by the known thickness. With the aid of a dye (such as trypan blue), it is possible to estimate cell viability. This is expressed as the percentage of viable cells determined as those with an intact membrane capable of excluding the dye from inside the cell and so remaining colorless (see Chapter 2 and Freshney, 2005).

Although counting by hemocytometer is the most common method for the determination of cell concentration, it does not allow any action to be taken during the culture process, because it is typically an offline measurement. When a real-time estimation is needed, it is common to use an indirect measurement, such as the optical density, or turbidity. It is possible to correlate the absorbance measured by a sensor introduced into the bioreactor with the cell concentration (Konstantinov *et al*., 1994; Wu *et al*., 1995; Olsson and Nielsen, 1997). Several sensors are commercially available for this function, notably those that measure reflected light (Aquasant AS81/44 and Metler FSC402) and transmitted light (ASR Laser Turbidimeter). Optical sensors can also be used for monitoring other variables, even in small bioreactors (Kostov *et al*., 2001).

Other forms of online estimation of the cell concentration are:

(i) A microscope directly inserted into the culture medium, using image analysis software to evaluate cell concentration and size distribution for each acquired image (Joeris *et al*., 2002; Gueza *et al*., 2004).
(ii) Capacitance measurement of the culture, that is correlated with the concentration of viable cells. Each cell with an intact membrane behaves as a small electric capacitor (Dowd *et al*., 2003).
(iii) Fluorescence measurement of the medium. This is based on the assumption of a constant content of a fluorophor such as NAD(P)H per cell.

As well as the estimation of the cell concentration, other parameters related to the biomass can be obtained. However, at present these are limited to offline measurements. Among these are:

(i) Distribution of the size of the cells, based on the Coulter Counter® (Beckman Coulter, USA) or similar equipment like Casy TTC (Innovatis, Germany), in which a dilute cell suspension flows through a capillary tube containing two electrodes. Each cell is detected by a disturbance in the electric current, the intensity of which is measured. In this way, not only are the cells counted, but size distribution is estimated, based on a precalibration relating the cell diameter to the disturbance of electric intensity (Seewöster and Lehmann, 1997). Digital image analysis based on equipment such as Cedex (Innovatis) also provides offline cell size distribution estimation.
(ii) Flow cytometry is a method through which it is possible to count cells presenting certain characteristics (Davey and Kell, 1996; Brezinsky *et al*., 2003). FACS (fluorescence activated cell sorter) is a variant of flow cytometry, in which besides counting each cell with certain characteristics (fluorescent), it is possible to separate them into subpopulations with similar characteristics (*Figure 10.4*; see color section) (Herzenberg *et al*., 2000; Park *et al*., 2006).

10.4.5 Other variables of interest

Besides the variables previously mentioned, the following deserve to be discussed.

(i) Partial pressure of dissolved carbon dioxide gas (pCO_2). Carbon dioxide is a compound present in the culture medium that can inhibit cell growth. In small-scale cultures (Schott or T-flasks), it is common to keep the flask in an incubator with a controlled CO_2 concentration. Such control is made through a sensor (infrared spectrophotometry or thermal conductivity) and a valve that releases carbon dioxide gas into the incubator. Through the presence of bicarbonate in the culture medium, it is possible to maintain a reasonably constant culture pH (see Section 10.4.2). In bioreactors, pCO_2 can be controlled through the addition of CO_2 to the sparged gas mixture. Cell growth inhibition will occur at very high or low pCO_2. Therefore, it is desirable to maintain its value between 40 and 200 mmHg, depending on the cell line. There are commercially available sensors designed for pCO_2, which can be sterilized and inserted into any bioreactor port, such as the InPro® 5000, with the principle of operation similar to a pH electrode (Mettler-Toledo, 2006). In the literature there are reports of sensors based on other technologies (Pattison *et al*., 2000).
(ii) Metabolites can be measured in real time, through techniques such as FIA (flow injection analysis) that allow online measurement of glucose and other substrates or products. In this system, a small flow of culture medium is continuously pumped to a unit composed of a tangential filter (for cell separation), injection pump, and detector (Reinecke and Stephanopoulos, 2000). Another possibility is to connect offline equipment to automated sampling systems (*Figure 10.5*), to provide information on the metabolite concentration for a control system, for instance, for controlling the flow of fresh medium in a fed-batch culture (Konstantinov *et al*., 1996; Ozturk *et al*., 1997). It is

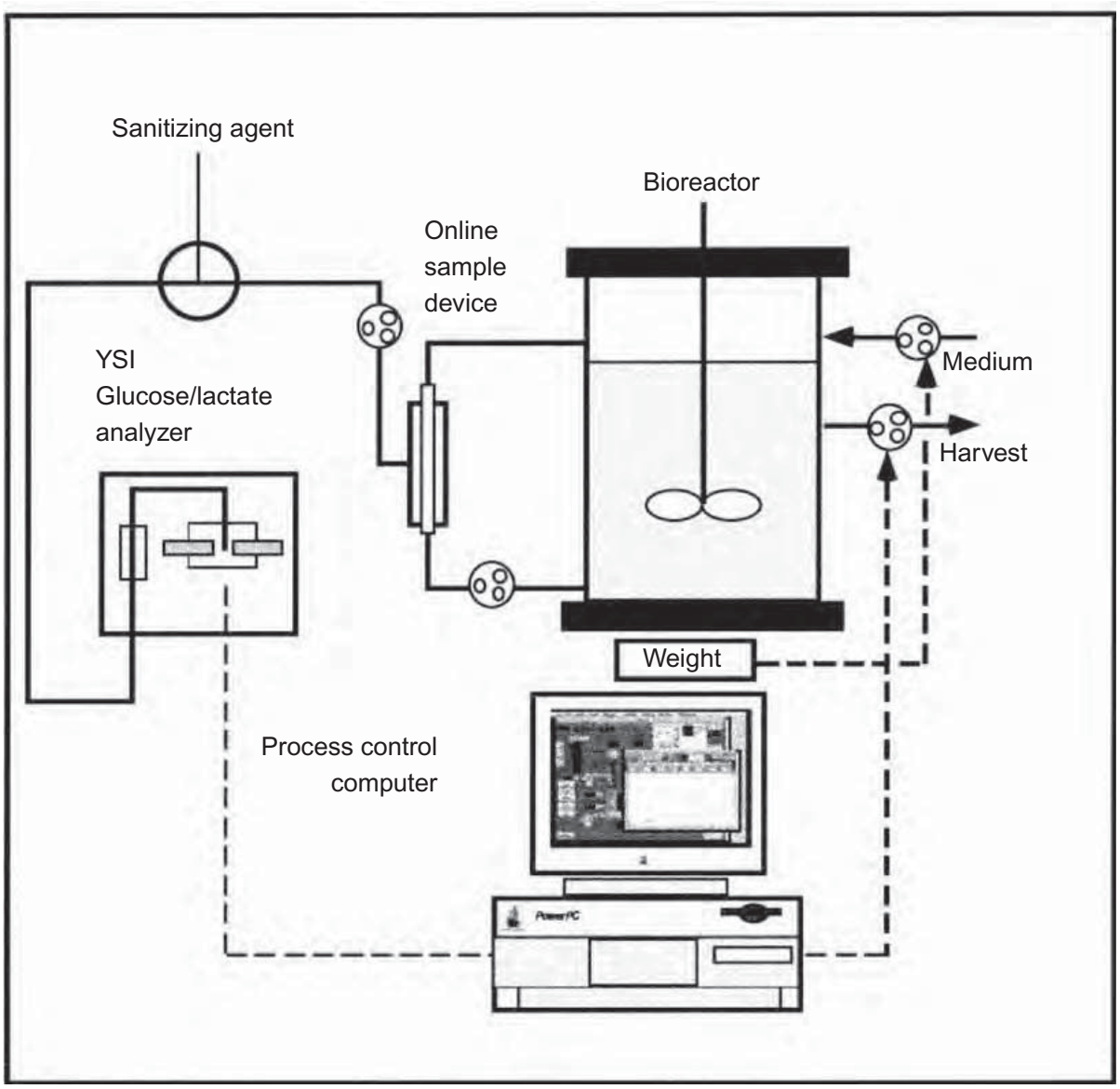


Figure 10.5

Feeding flow rate control system based on glucose concentration measured in real time (adapted from Ozturk *et al.*, 1997).

also possible to estimate cell concentration, including those immobilized on microcarriers based on measurements of real-time substrate consumption (Ducommun *et al.*, 2001).

10.5 Strategies of control

10.5.1 Traditional control

There are several strategies to control a process variable. Among them, the most important are the following.

(i) On-off controller, in which the action can only assume two states (on or off). This controller is used, for instance, in incubators to keep temperature in a small range. When the temperature sensor indicates a value lower than set up, the system turns an electric resistance on, heating up its interior. In this situation, it is not possible to take an action larger or smaller, that means, it is warming up or it is not.

(ii) Controller modulated by pulse width (pulse width modulation or PWM). In this control type, the action is also on or off but the time that the actuator stays on within a certain cycle can be adjusted continuously, allowing a final operation of different intensities. Frequently the pH control in bioreactors uses this strategy. The greater the difference between the pH set-point and the measured value, the larger would be the fraction of the cycle (usually a few seconds) in which the base pump is on, creating prolonged action within the cycle. This strategy prevents an exaggerated control action that would cause substantial deviation from the set-point value (called overshoot). Some dissolved oxygen control systems operate this way, through the controlled mixing of different gases by the opening and closing of solenoid valves.

(iii) Cascade controller, composed of one master and one slave loop. This is used when a more rigid control of a process variable is required, for instance, the temperature of the culture medium. Here the action of the master controller (primary loop) to keep bioreactor temperature controlled is simply to calculate the adequate value for the slave controller set-point, which takes action in the process (*Figure 10.6*).

(iv) PID or proportional integral derivative controller. This is the most common system used in industry and the one that best keeps control of the process variable. It is based on the principle that the action is taken not only on how large is the error (difference between desired and measured values), but also on the sum of past errors (integral of

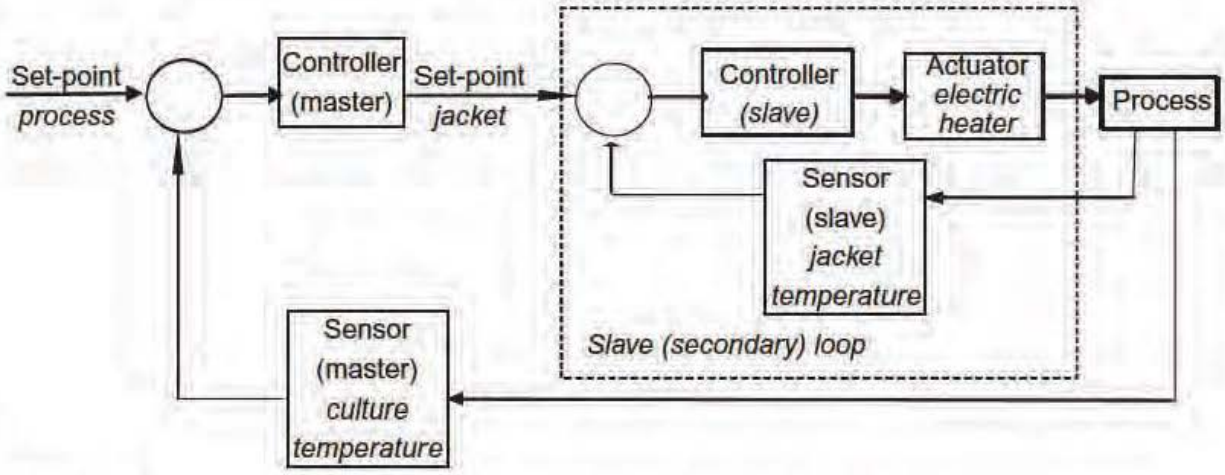


Figure 10.6

Cascade control. The actuator of the slave controller (electric heater) maintains the temperature of the jacket water controlled in a value set by the master controller.

the error) and to the rate that the error is changing (derivative of the error). Mathematically it can be expressed as in Equation 3:

$$\text{actuation} = \text{P.error} + \text{I.}\int \text{error.dt} + \text{D.}\frac{\text{d error}}{\text{dt}} \quad (3)$$

where actuation is the controller output (the value that goes to the actuator, according to *Figure 10.1*), error is the difference between the desired value (set-point) and the one measured by the sensor, t is time and P, I, and D are constants that need to be adjusted for each system. The adjustment of the constants for a process, that is, the algorithm to determine the numeric values that will make the controller operate better, is called PID controller tuning. The most common procedure for this is called Ziegler-Nichols. For further details to tune a controller for a given process, a textbook of process control is recommended (Seborg *et al*., 2003).

10.5.2 Advanced control

For chemical processes (without cells) several advanced control strategies have been developed, some of which can be applied to bioprocesses and, especially, in animal cell cultures. Two of them are described below.

Adaptive control. When there are big changes during a process, a certain set of values of constants like P, I, and D, may not be adequate for the whole culture. The controller may be tuned, for instance, to the beginning of the culture, but not to the end. In this situation, the controlled variable may start to oscillate significantly, which can interfere with the process. For those cases, algorithms have been developed to tune these constants during the process. Such a procedure is called adaptive control (Diaz *et al*., 1996).

Control based on neural network. Similar to fuzzy logic modeling, neural network analysis uses a series of previous data to execute simulations of the process, with a high degree of success, without however using formal mathematical models (Chen and Rollins, 2000). To this goal, it is necessary to define inputs, outputs, and how many layers of ''neurons'' will be used, which depends on the number of variables and the available data.

References

Bailey JE, Ollis DF (1986), Biochemical engineering fundamentals, 2nd Ed., McGraw Hill, Singapore.

Brezinsky SCG, Chiang GG, Szilvasi A, Mohan S, Shapiro RI, MacLean A, Sisk W, Thill G (2003), A simple method for enriching populations of transfected CHO cells for cells of higher specific productivity, J. Immunol. Methods 277:141 155.

Chen VCP, Rollins DK (2000), Issues regarding artificial neural network modeling for reactors and fermenters, Bioprocess. Eng. 22:85 93.

Cherlet M, Marc A (1998), Intracellular pH monitoring as a tool for the study of hybridoma cell behavior in batch and continuous bioreactor cultures, Biotechnol. Prog. 14:626 638.

Chuppa S, Tsai YS, Yoon S, Shackleford S, Rozales C, Bhat R, Tsay G, Matanguihan

C, Konstantinov K, Naveh D (1996), Fermentor temperature as a tool for control of high density perfusion cultures of mammalian cells, Biotechnol. Bioeng. 55:328 338.
Davey HM, Kell DB (1996), Flow cytometry and cell sorting of heterogeneous microbial populations: the importance of single cell analyses, Microbiol. Rev. 60:641 696.
Diaz C, Dieu P, Feuillerat C, Lelong P, Salome M (1996), Simultaneous adaptive predictive control of the partial pressures of dissolved oxygen (pO2) and dissolved carbon dioxide (pCO2) in a laboratory scale bioreactor, J. Biotechnol. 52: 135 150.
Dowd JE, Jubb A, Kwok KE, Piret JM (2003), Optimization and control of perfusion cultures using a viable cell probe and cell specific perfusion rates, Cytotechnology 42:35 45.
Ducommun P, Bolzonella I, Rhiel M, Pugeaud P, von Stockar U, Marison IW (2001), On line determination of animal cell concentration, Biotechnol. Bioeng. 72: 515 522.
Fox SR, Patel UA, Yap MG, Wang DI (2004), Maximizing interferon gamma produc tion by Chinese hamster ovary cells through temperature shift optimization: experimental and modeling, Biotechnol. Bioeng. 85:177 184.
Freshney RI (2005), Culture of Animal Cells: A Manual of Basic Technique, 5th Ed., Wiley Liss, New York.
Gueza JS, Cassar JP, Wartelle F, Dhulster P, Suhr H (2004), Real time in situ microscopy for animal cell concentration monitoring during high density culture in bioreactor, J. Biotechnol. 111:335 343.
Henzler HJ, Kauling DJ (1993), Oxygenation of cell cultures, Bioprocess Eng. 9: 61 75.
Herzenberg LA, De Rosa SC, Herzenberg LA (2000), Monoclonal antibodies and the FACS: complementary tools for immunobiology and medicine, Immunol. Today 21:383 390.
Joeris K, Frerichs J G, Konstantinov K, Scheper T (2002), In situ microscopy: online process monitoring of mammalian cell cultures, Cytotechnology 38:129 134.
Johnson MJ, Borkowski J, Engblom C (2000), Steam sterilizable probes for dissolved oxygen measurement, Biotechnol. Bioeng. 67:645 656.
Jorjani P, Ozturk SS (1999), Effects of cell density and temperature on oxygen consumption rates for different mammalian cell lines, Biotechnol. Bioeng. 64: 349 356.
Kamen AA, Bédard C, Tom R, Perret S, Jardin B (1996), On line monitoring of respiration in recombinant baculovirus infected and uninfected insect cell bior eactor cultures, Biotechnol. Bioeng. 50:36 48.
Kilburn DG (1991), Monitoring and control of bioreactors, In: Butler M (Ed.) Mammalian Cell Biotechnology. A Practical Approach, Oxford University Press, New York, pp. 159 185.
Konstantinov K, Chuppa S, Sajan E, Tsai Y, Yoon S, Golini F (1994), Real time biomass concentration monitoring in animal cell cultures, Trends Biotechnol. 12:324 333.
Konstantinov KB, Tsai Y, Moles D, Matanguihan R (1996), Control of long term perfusion Chinese Hamster Ovary cell culture by glucose auxostat, Biotechnol. Prog. 12:100 109.
Kostov Y, Harms P, Randers Eichhorn L, Rao G (2001), Low cost microbioreactor for high throughput bioprocessing, Biotechnol. Bioeng. 72:346 352.
Mettler Toledo (2006), Available at: http://www.mt.com (accessed 20 December 2006).
Moore A, Donahue CJ, Hooley J, Stocks DL, Bauer KD, Mather JP (1995), Apoptosis in CHO cell batch cultures: examination by flow cytometry, Cytotechnology

17:1 11.
Olsson L, Nielsen J (1997), On line and in situ monitoring of biomass in submerged cultivations, Trends Biotechnol. 15:517 522.
O'Neal DP, Meledeo MA, Davis JR, Ibey BL, Gant VA, Pishko MV, Coté GL (2004), Oxygen sensor based on the fluorescence quenching of a ruthenium complex immobilized in a biocompatible poly(ethylene glycol) hydrogel, IEEE Sensors Journal 4:728 734.
Ozkan P, Mutharasan R (2002), A rapid method for measuring intracellular pH using BCECF AM, Biochim. Biophys. Acta 1572:143 148.
Ozturk SS, Thrift JC, Blackie JD, Naveh D (1997), Real time monitoring and control of glucose and lactate concentrations in a mammalian cell perfusion reactor, Biotechnol. Bioeng. 53:372 378.
Park JY, Lim B P, Lee K, Kim Y G, Jo E C (2006), Scalable production of adeno associated virus type 2 vectors via suspension transfection, Biotechnol. Bioeng. 94:416 430.
Pattison RN, Swamy J, Mendenhall B, Hwang C, Frohlich BT (2000), Measurement and control of dissolved carbon dioxide in mammalian cell culture processes using an in situ fiber optic chemical sensor, Biotechnol. Prog. 16:69 74.
Qi HN, Goudar CT, Michaels JD, Henzler H J, Jovanovic GN, Konstantinov KB (2003), Experimental and theoretical analysis of tubular membrane aeration for mammalian cell bioreactors, Biotechnol. Prog. 19:1183 1189.
Reinecke M, Stephanopoulos G (2000), Flow injection analysis for simultaneous quantification of prolactin concentration and glycosylation macroheterogeneity in cell culture samples, Cytotechnology 34:237 242.
Ruffieux P, Stockar U, Marison IW (1998), Measurement of volumetric (OUR) and determination of specific (qO2) oxygen uptake rates in animal cell cultures, J. Biotechnol. 63:85 95.
Schügerl K (Ed.) (1991), Measuring, Modelling and Control, vol. 4, 2nd Ed., In: Rehm HJ, Reed G (Eds), Biotechnology, VCH, Weinheim.
Schügerl K (2001), Progress in monitoring, modeling and control of bioprocesses during the last 20 years, J. Biotechnol. 85:149 173.
Seborg DE, Edgar TF, Mellichamp DA (2003), Process Dynamics and Control, 2nd Ed., Wiley, New York.
Seewöster T, Lehmann J (1997), Cell size distribution as a parameter for the predeter mination of exponential growth during repeated batch cultivation of CHO cells, Biotechnol. Bioeng. 55:793 797.
Sonnleitner B (2000), Instrumentation of biotechnological processes, Advances in Biochemical Engineering Biotechnology, vol. 66, Springer, Berlin, pp. 1 64.
Wu P, Ozturk SS, Blackie JD, Thrift JC, Figueroa C, Naveh D (1995), Evaluation and applications of optical cell density probes in mammalian cell bioreactors, Biotech nol. Bioeng. 45:495 502.
Yoon S, Konstantinov KB (1994), Continuous, real time monitoring of the oxygen uptake rate (OUR) in animal cell bioreactors, Biotechnol. Bioeng. 44:983 990.

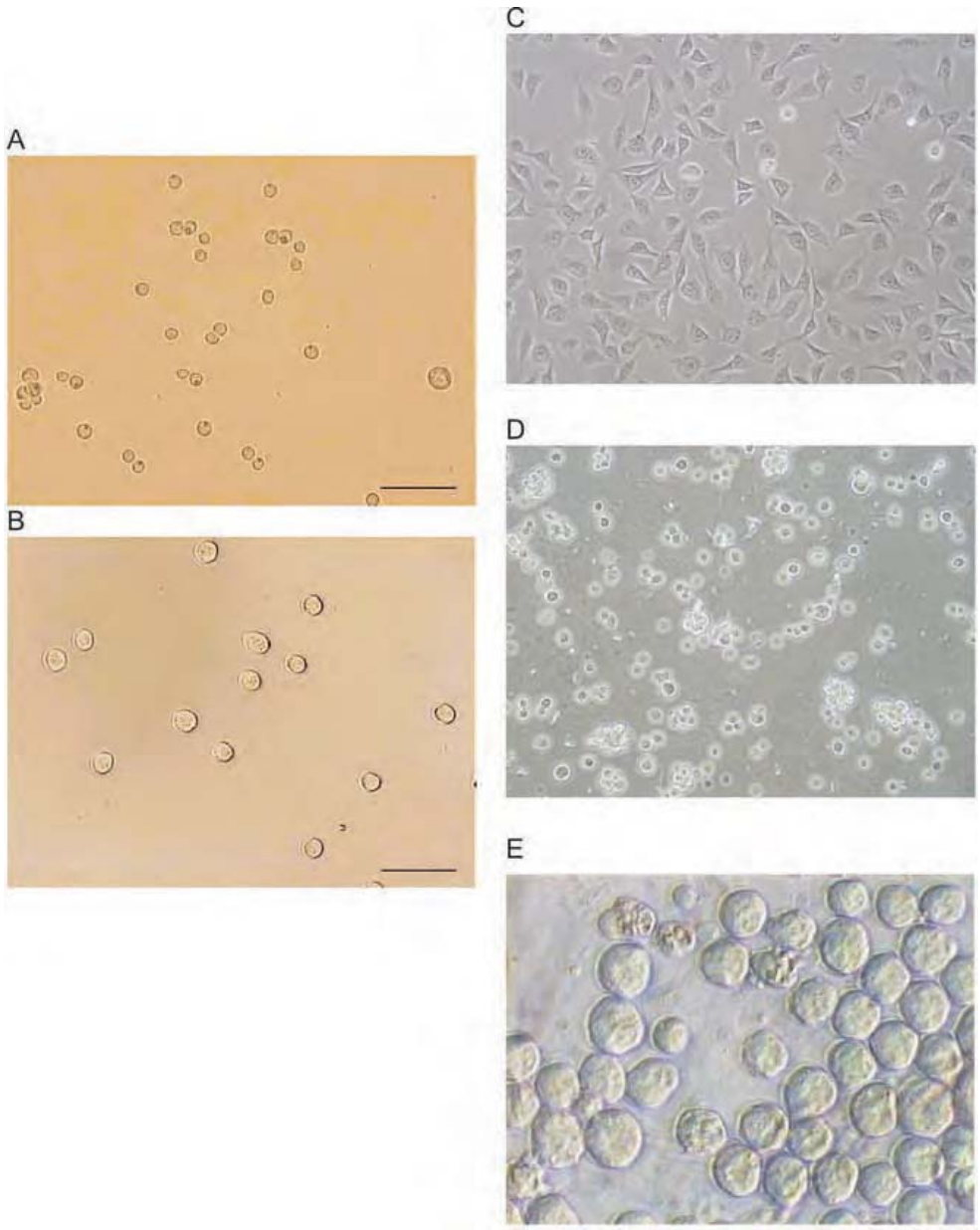


Figure 2.4

Different morphology types observed in distinct cell lines. (A) *Drosophila melanogaster* cell line S2; (B) *Spodoptera frugiperda* cell line Sf9; (C) adherent CHO.K1 cell line; (D) CHO.K1 cell line adapted to suspension growth; (E) hybridoma producing anti TNP (trinitrophenol) monoclonal antibody. Bars in photos (A) and (B) represent 50 μm. Photographs (C) and (D) were kindly provided by Rodrigo Coelho Ventura Pinto (Cell Culture Engineering Laboratory, COPPE, Federal University of Rio de Janeiro, Brazil), and photograph (E) was kindly provided by Rita de Cássia Paro Alli (Industrial Biotechnology Laboratory, IPT, São Paulo, Brazil).

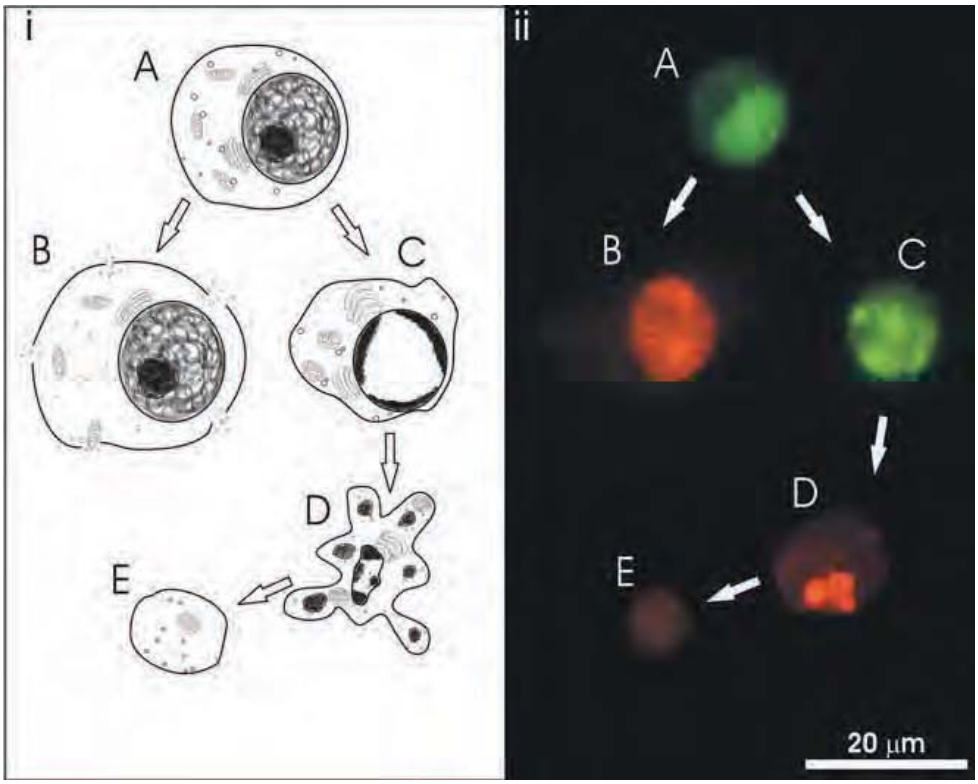


Figure 7.3

Morphological changes in apoptosis and necrosis. (i) A viable nonapoptotic cell (A), in case of death by necrosis, undergoes a cell swelling process, followed by cell lysis and release of intracellular material (B). In case of apoptosis, chromatin condensation near the nuclear membrane (C) occurs and apoptotic bodies (D) are formed. A final stage, where the cell presents no chromatin (E), may also be observed. Under *in vitro* culture conditions, a phenomenon known as secondary necrosis occurs in the final stages of apoptosis. (ii) Fluorescence microscopy using acridine orange and ethidium bromide allows identification of the different stages of cell death: viable nonapoptotic cells (A), necrotic cells (B), viable apoptotic cells (C), nonviable apoptotic cells (D), and chromatin free cells (E).

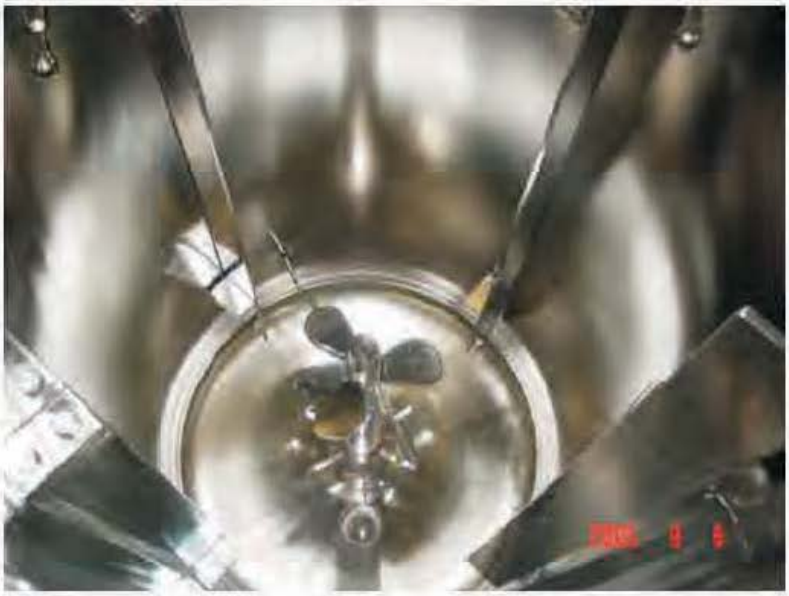


Figure 9.4

Internal surface of a 1000 L bioreactor for animal cell cultivation. It is also possible to observe the three blade impeller and the baffles, which are flat vertical parts attached to the internal bioreactor surface with the aim of avoiding vortex formation.

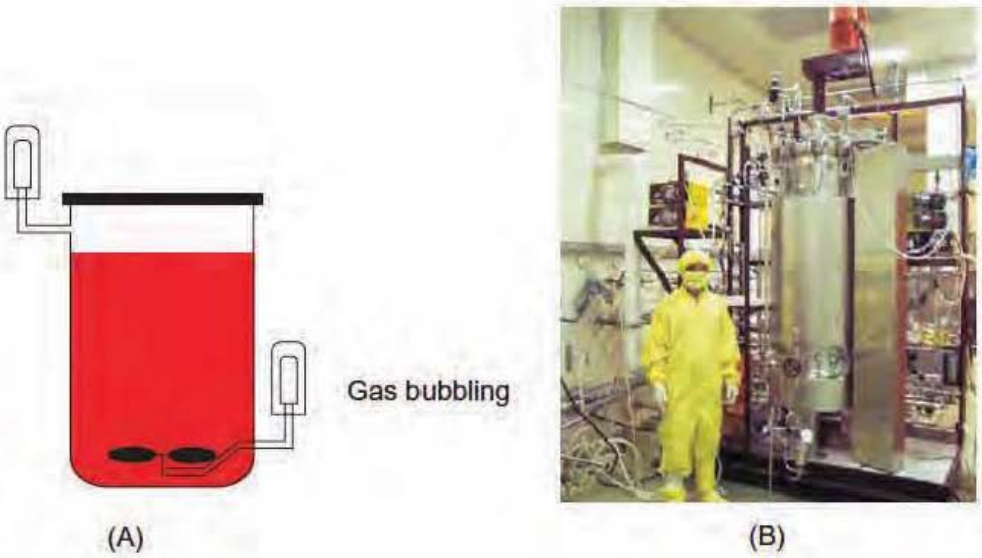


Figure 9.5

Stirred tank bioreactor with a bubble aeration system: (A) schematic representation; (B) photograph of a 300 L bioreactor (CIM, Cuba).

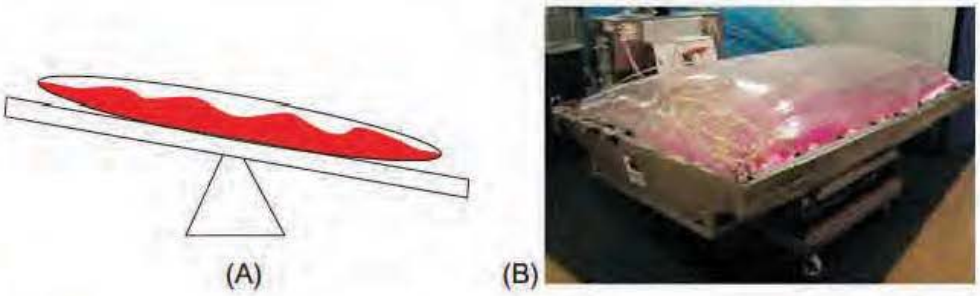


Figure 9.6

Wave bioreactor: (A) schematic representation; (B) photograph of a 500 L bioreactor during a conference exhibition of the company Wave Biotech.

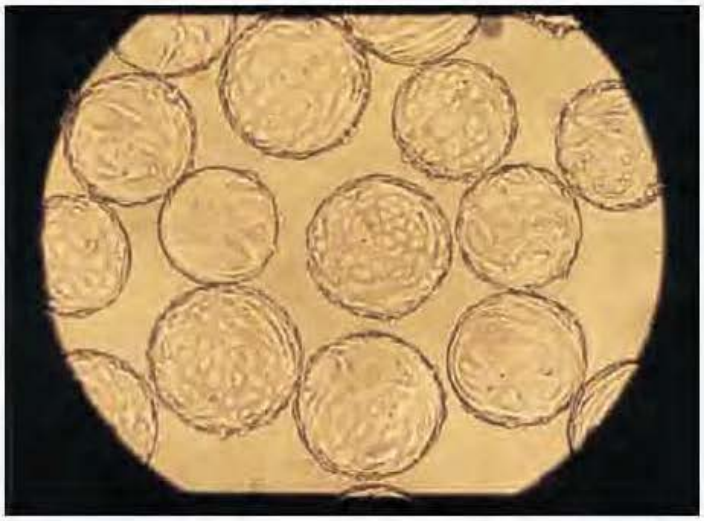


Figure 9.8

Photograph taken under an optical microscope of Vero cells growing on microporous microcarriers.

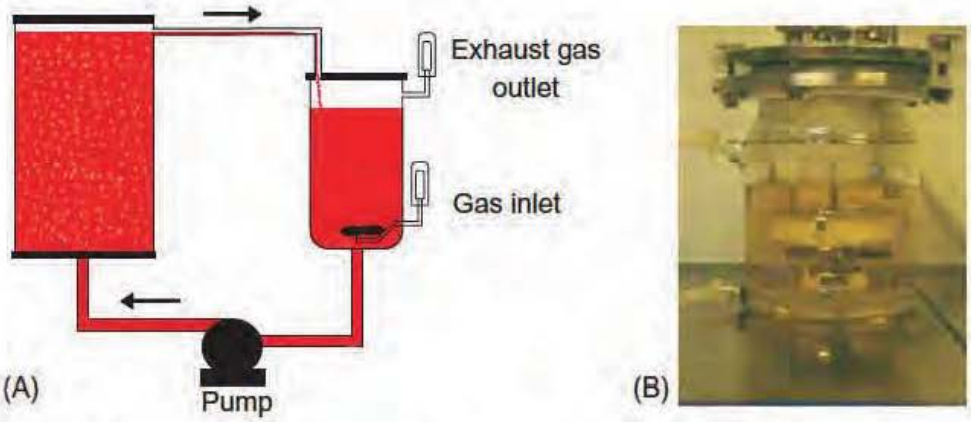
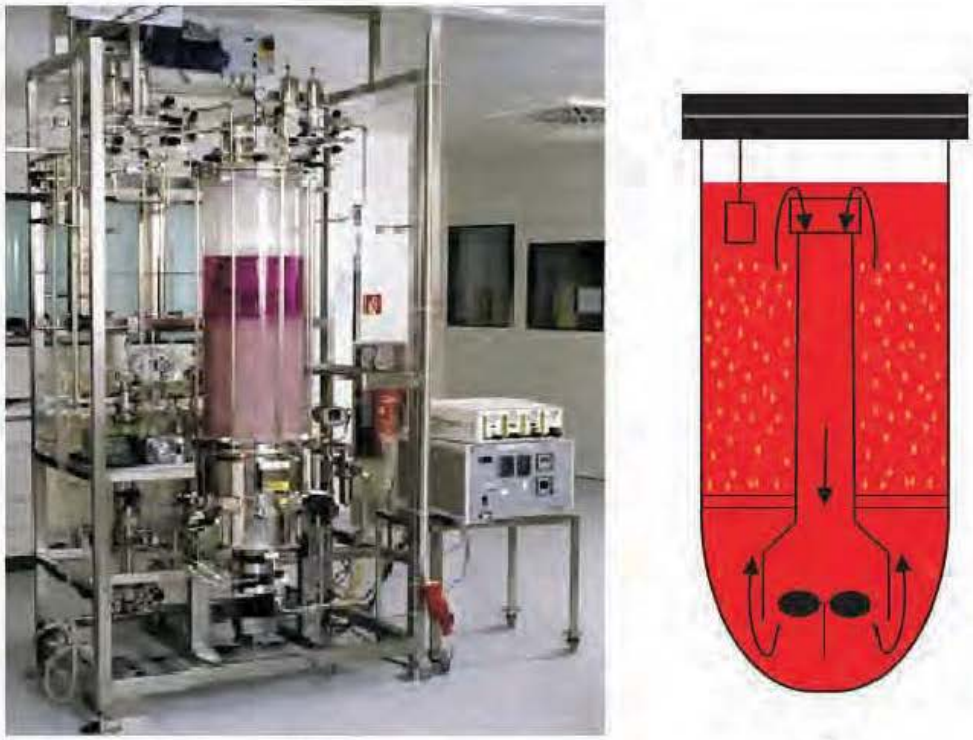


Figure 9.9

Fixed bed bioreactor: (A) schematic representation including the external recirculation loop with an aeration tank; (B) photograph of a small scale bioreactor.



(A) (B)

Figure 9.10

Fluidized bed bioreactor: (A) photograph of a bioreactor commercialized by GE Healthcare; (B) operational scheme.

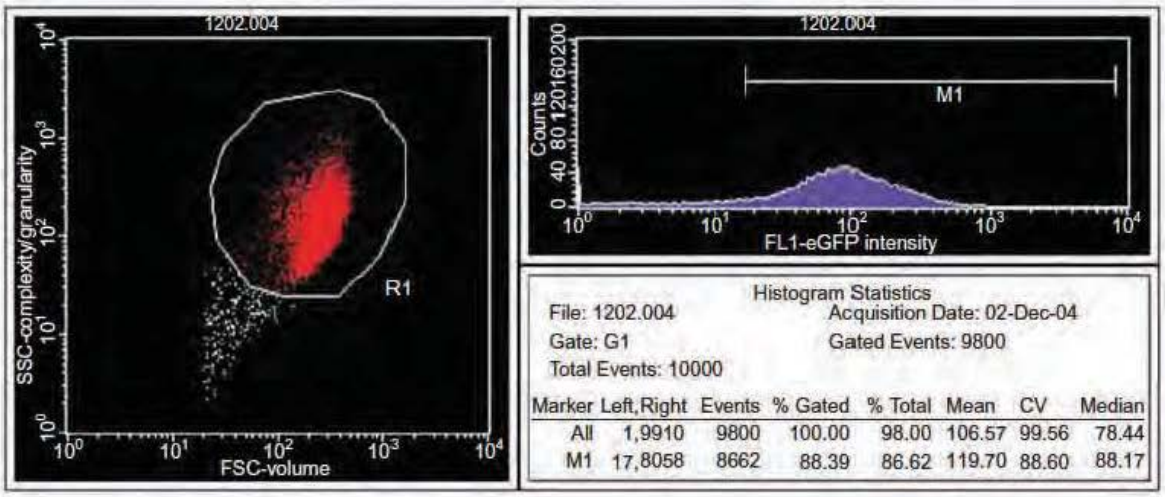


Figure 10.4

Example of insect cell analysis that expresses the GFP (green fluorescent protein), through a FACS machine. (A) The abscissa indicates the front dispersion (frontal scatter count, or FSC), that evaluates the cell volume (size), while the ordinate shows the lateral light dispersion of each cell (side scatter count, or SSC), that estimates the intracellular granularity (particles and intracellular organelles). As each cell is positioned in part of the dispersion, it is possible to quantify their subpopulations. (B) The histogram shows the distribution of the number of cells for each dispersed light intensity. If a limit is defined, population fractions are established (reprinted by permission from Mariza A. Gerdulo dos Santos).

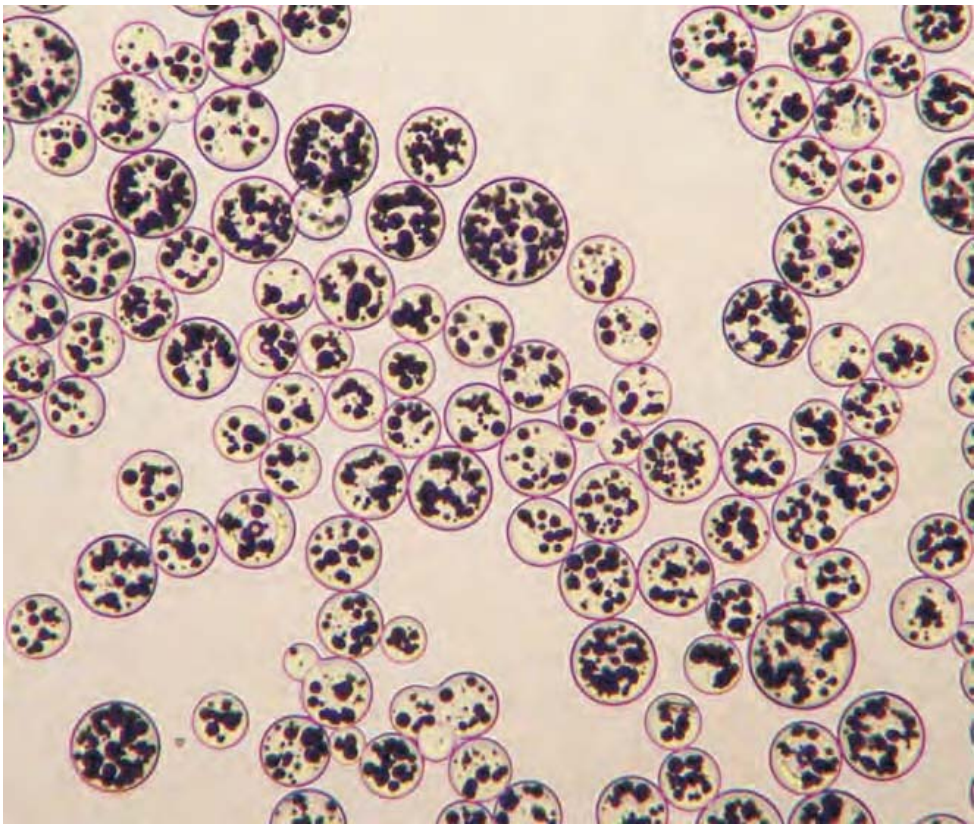


Figure 12.7

Optical microscope photo of Streamline rPrA® resin, showing the adsorbent particles composed of quartz and cross linked agarose in different proportions.

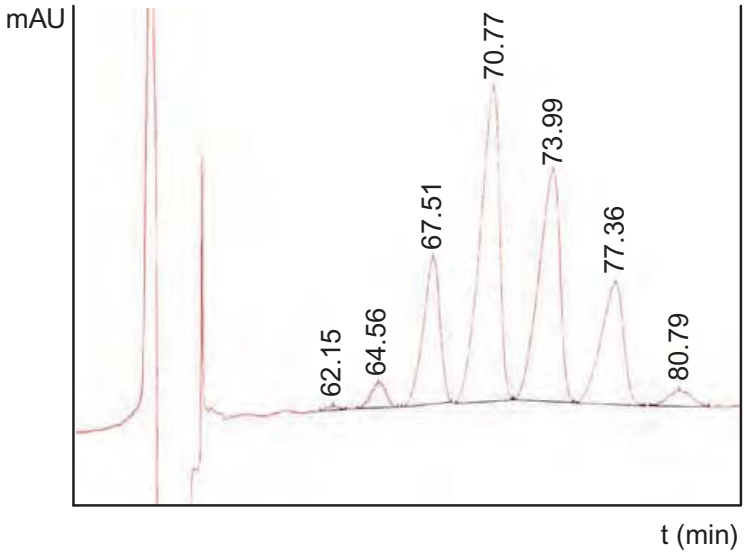


Figure 13.4

Capillary electrophoresis of purified rhEPO (recombinant human erythropoietin) protein. From left to right: isoform nos 2, 3, 4, 5, 6, 7, and 8 (according to *European Pharmacopea*).

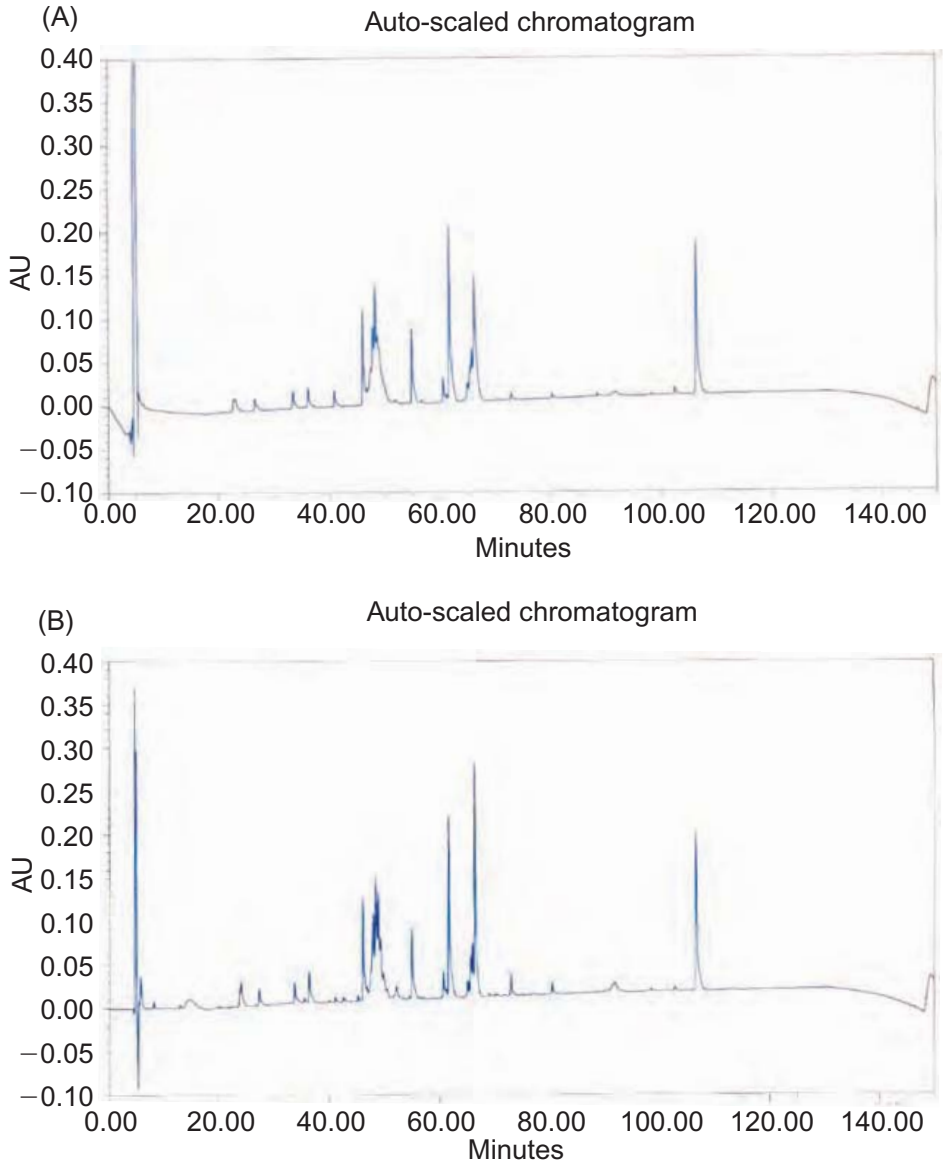


Figure 13.1

Peptide mapping. (A) Peptide mapping of the international standard of rhEPO (recombinant human erythropoietin) (BRP *European Pharmacopea*). (B) Peptide mapping of an rhEPO production lot.

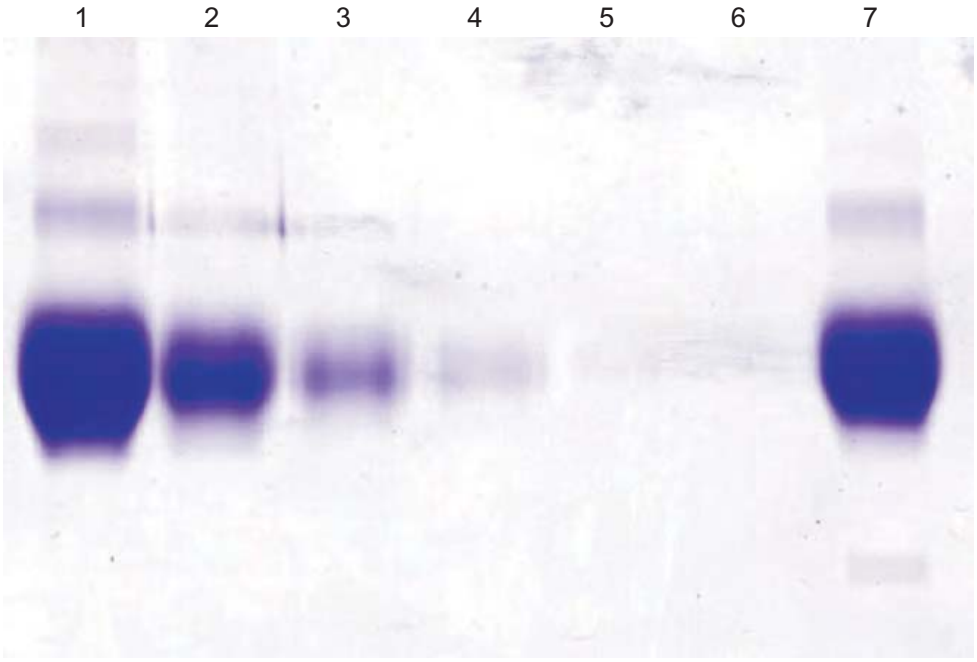


Figure 13.2

SDS PAGE of purified rhEPO (recombinant human erythropoietin). Lanes 1 5: dilutions 1: 5 of product (25, 5, 1, 0.2, and 0.04 μg, respectively); lane 7: international standard (25 μg).

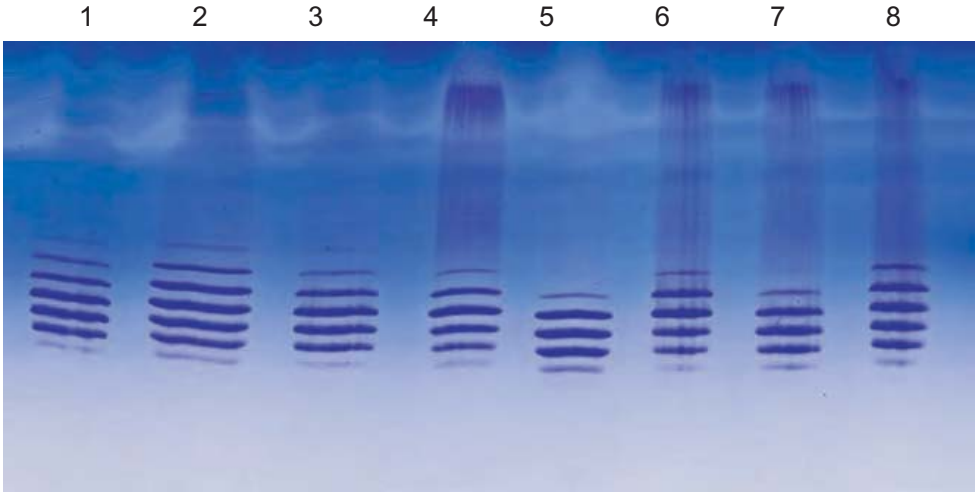


Figure 13.3

Isoelectric focusing of different samples of rhEPO (recombinant human erythropoietin).The different profiles of isoforms are observed in the different preparations. Lane 3: international standard.

Animal cell separation

11

Leda dos Reis Castilho and Ricardo de Andrade Medronho

11.1 Introduction

After cell cultivation, a cell separation step is generally needed to process the culture medium to purify the product. This step is performed either to obtain a cell-free medium for an extracellular product or to obtain a cell concentrate if the product is intracellular. In animal cell cultures, the product is normally secreted. Thus, after cell separation, the product is in solution and can be processed by chromatographic techniques or other protein purification methods, as presented in Chapter 12.

When the culture is operated in perfusion mode (Chapter 9), the retention device may be placed inside or outside the bioreactor (*Figure 11.1*). When the cell culture is operated in batch or fed-batch mode, the cell separation is conducted after the end of the cultivation period (*Figure 11.2*). This is the first step of downstream processing.

Animal cell separation is far from a trivial task for many reasons. Animal cells have no cell wall. They are protected only by the cellular membrane (lipid bilayer). For this reason, they present high sensitivity to shear stress. When cultivated in suspension, the cells tend to attach to the device walls, which may generate clogging problems. Their density is not much higher than water (1.06–1.14 g cm ^{3}) and cell size is in the range of 8–40 μm, which results in low settling velocities (Medronho, 2003). For example, Luebberstedt (2000) worked with HeLa cells with a density of 1.06 g cm ^{3} and varying from 10 to 28 μm in size. Schultz (1996) found BHK-21 cell sizes in the 11–25 μm range.

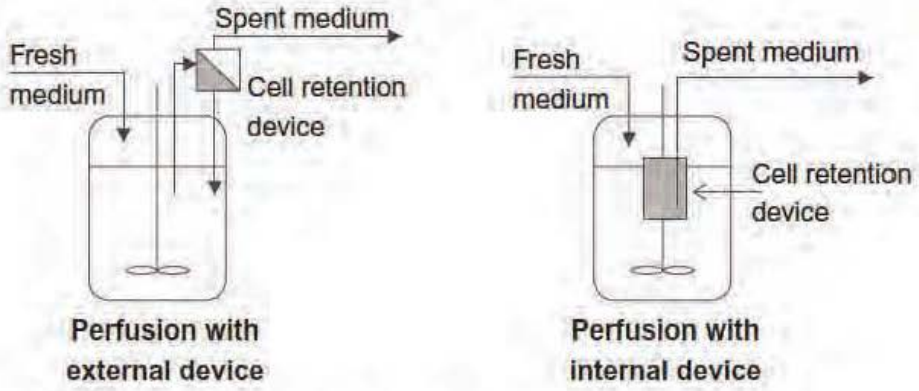


Figure 11.1

External (left) and internal (right) cell separation device in a perfusion culture.

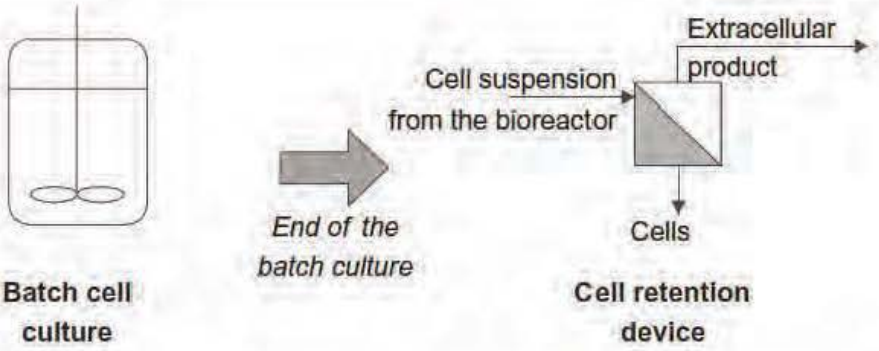


Figure 11.2

Cell separation device as the first step of the downstream processing in a batch culture.

The terminal settling velocity (v_t) of a spherical particle under laminar flow can be described by Stokes equation (Equation 1):

$$v_t = \frac{(\rho_s - \rho)\, b\, d^2}{18\,\mu_L} \tag{1}$$

where ρ_s and ρ are the particle (cell) density and the fluid (medium) density, respectively; b is the force field intensity ($b = g$ for a gravitational field or $b = \omega^2 r$ for a centrifugal field); d is the particle (cell) diameter; μ_L is the fluid viscosity; g is the acceleration due to gravity (9.81 m s ^{2}); ω is the angular velocity; and r is the radial distance of the particle (cell) from the rotation axis.

Terminal settling velocities decrease with cell concentration. Therefore, for cell suspensions with concentrations higher than 1% by volume (around 6×10^6 cells mL ^{1} for cells with 15 μm diameter), the terminal settling velocity given by Equation (1) should be corrected using Equation (2). This equation is valid under laminar flow (Stokes region):

$$v_{tc} = v_t(1 - c_v)^{4.65} \tag{2}$$

where c_v is the concentration by volume; v_{tc} is the terminal settling velocity of the cell at a given concentration c_v, and v_t is the terminal settling velocity of the cell given by equation (1).

This chapter describes the main unit operations employed for animal cell separation. These are gravity settlers, centrifuges, hydrocyclones, filters, and ultrasonic separators.

11.2 Separation efficiency

Monitoring the separation efficiency is an important way to evaluate the performance of a given separator. The efficiency concepts described in this section apply not only to animal cell separation, but also to the separation of any particles and bioparticles, such as microorganisms, cellular debris, nuclei, etc. These concepts apply to any separation device whose performance remains constant if the operational conditions do not change. This happens, for instance, in gravity settlers, centrifuges, and hydrocyclones.

Two kinds of separation efficiency may be defined when dealing with separation devices: the total and the grade efficiency. The total efficiency is a global efficiency and the grade efficiency is the efficiency attained for a given cell size. To obtain the grade efficiency it is necessary to know the cell size distributions. These distributions may be presented either as frequency x or as cumulative distributions (undersize y or oversize z). *Figure 11.3* shows the frequency plot (*Figure 11.3A*) and the cumulative plots (*Figure 11.3B*) of HeLa cells cultivated in a stirred bioreactor, in a serum-free medium (Luebberstedt, 2000).

These three forms of presenting the cell size distribution are interrelated through Equations (3) and (4).

$$x = \frac{dy}{dd} \tag{3}$$

$$y = 1 - z \tag{4}$$

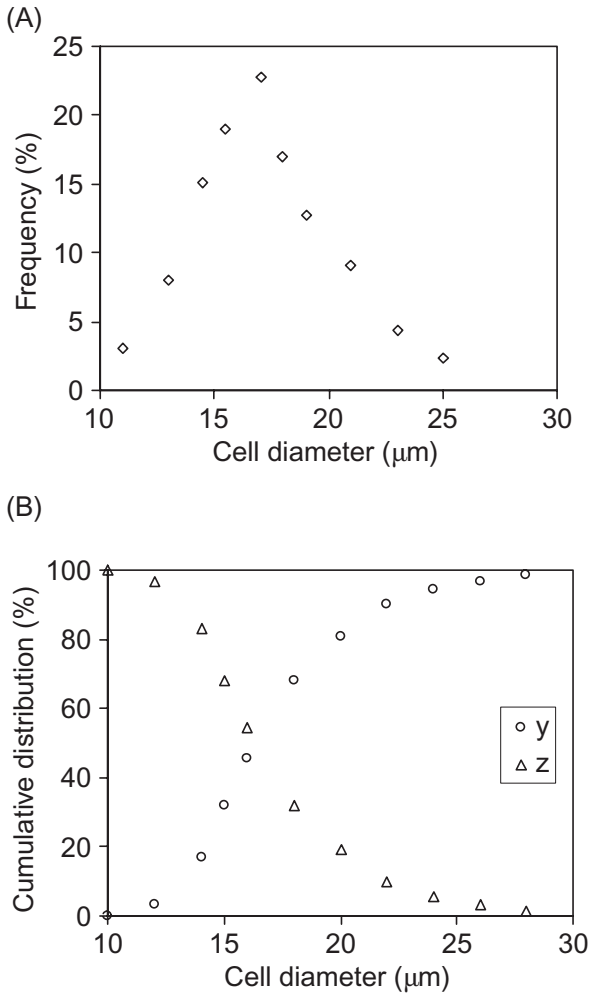


Figure 11.3

Cell size distribution of HeLa cells grown in serum free culture medium (Luebberstedt, 2000), presented as (A) frequency and (B) cumulative undersize (y) and oversize (z) distributions.

A given separator has three main streams: the feed, which carries the cells to be separated, the stream concentrated in cells (underflow), and the diluted stream (overflow), containing the cells that the separator was not able to capture. These three streams are shown in *Figure 11.4*.

The total separation efficiency E is the cell fraction recovered in the concentrated stream, as given by Equation (5).

$$E = \frac{Q_C X_C}{Q X} \tag{5}$$

where Q and Q_C are the flow rates and X and X_C are the cell concentrations, as cell number per unit volume, of the feed and concentrated streams, respectively.

The fluid fraction discharged in the concentrated stream is known as the flow ratio R_f. For diluted suspensions like the ones usually found in cell cultures, R_f may be expressed by Equation (6). Some separators operate with $R_f = 0$ and some with $R_f > 0$. Because the fluid leaving the separator in the concentrated stream drags some particles with it, these particles are separated not due to the separation power of the separator but due to the drag force. This bypass is normally considered to be equal to the flow ratio (Medronho, 2003). Hence, R_f also represents the lowest separation efficiency at which the separator will operate.

$$R_f = \frac{Q_C}{Q} \tag{6}$$

The reduced total efficiency E' (Equation 7) is normally used for separators that operate with $R_f > 0$. This gives the separation efficiency of the particles that are separated in the concentrated stream only due to the separating power of the separator. Therefore, E' does not consider the particles that reach the underflow due to drag.

$$E' = \frac{E - R_f}{1 - R_f} \tag{7}$$

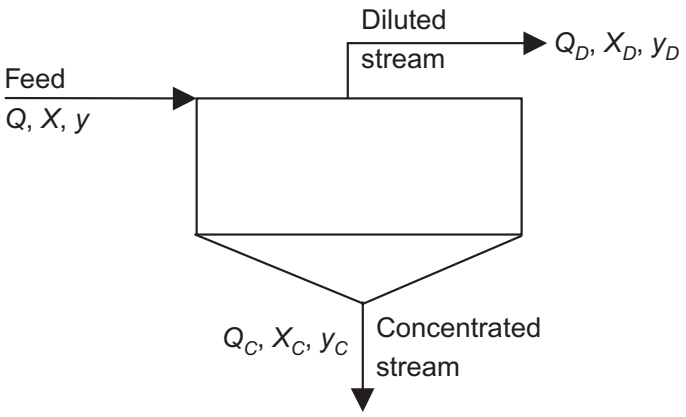


Figure 11.4

Schematic drawing of a separator showing the flow rates *Q*, the cell concentrations *X*, and the cumulative undersize distributions *y*.

Based on Equations (5), (6), and (7) and in material balances for the liquid and for the cells, it is possible to obtain Equation (8).

$$E' = 1 - \frac{X_D}{X} \tag{8}$$

The definition of the grade efficiency (Equation 9) is similar to the total efficiency definition, but it applies only to a given bioparticle size. *Figure 11.5* shows a typical grade efficiency curve:

$$G = \frac{Q_C X_{dC}}{Q X_d} \tag{9}$$

where G is the grade efficiency, and X_{dC} and X_d are the concentrations of cells of size d in the concentrated and feed streams, respectively.

Based on Equations (3) and (5), it is possible to rewrite Equation (9) as:

$$G = E\left(\frac{d\,y_C}{d\,y}\right) \tag{10}$$

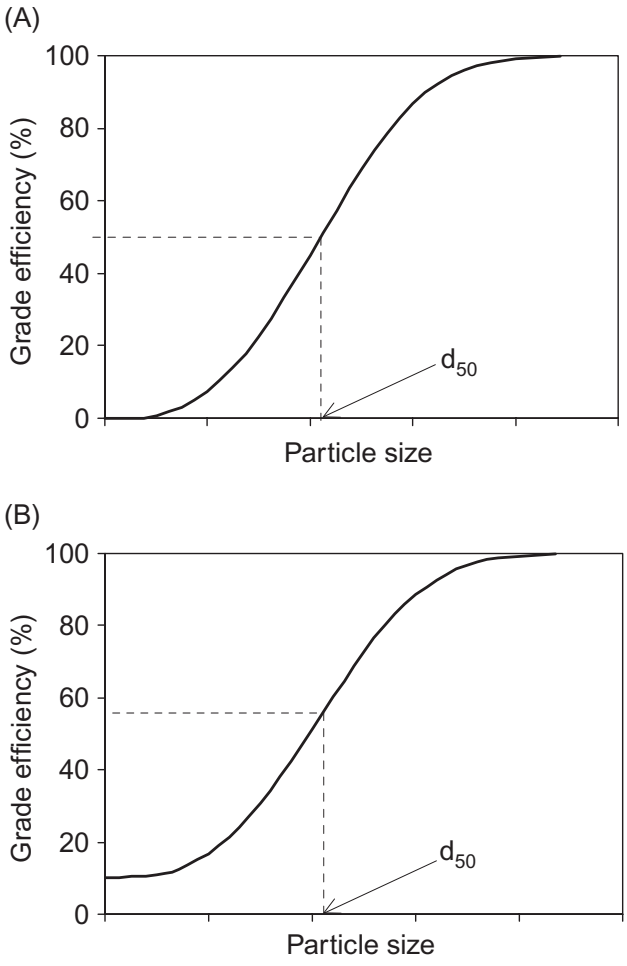


Figure 11.5

Grade efficiency curve for R_f equal to 0% (A) and greater than 0% (B).

Equation (11) represents a global mass balance for the cells of diameter d.

$$QX_d = Q_C X_{dC} + Q_D X_{dD} \tag{11}$$

Based on Equations (9) and (11), it is possible to write:

$$G = 1 - \left(\frac{Q_D X_{dD}}{Q X_d}\right) \tag{12}$$

Therefore, based on Equations (3) and (5):

$$G = 1 - (1 - E)\frac{d\,y_D}{d\,y} \tag{13}$$

From Equation (11), it is possible to write:

$$QX\left(\frac{d\,y}{d\,d}\right) = Q_C X_C\left(\frac{d\,y_C}{d\,d}\right) + Q_D X_D\left(\frac{d\,y_D}{d\,d}\right) \tag{14}$$

Dividing Equation (14) by QX and using Equation (10):

$$\frac{1}{G} = 1 + \left(\frac{1}{E} - 1\right)\left(\frac{d\,y_D}{d\,y_C}\right) \tag{15}$$

Through Equations (10), (13), and (15), it is possible to obtain the grade efficiency curve based on the total efficiency E and the size distributions of two out of three streams (y, y_D and y_C).

The cell size that is separated with 50% grade efficiency (d_{50}) is usually accepted as the cut size (*Figure 11.5*). This is an indirect measure of the separation power of a separator device. A better designed separator will give a lower cut size. A high proportion of the cells smaller than d_{50} will leave the separator in the diluted stream while the majority of those greater than d_{50} will leave it in the concentrated stream.

For separators which operate with $R_f > 0$, the grade efficiency curve does not start at $G = 0\%$ (*Figure 11.5B*). This is because the separator operation always gives a minimum efficiency almost equal to the flow ratio R_f. The reduced grade efficiency G' may be obtained in the same way as the reduced total efficiency E':

$$G' = \frac{G - R_f}{1 - R_f} \tag{16}$$

A chart showing the reduced grade efficiency versus cell size starts in the origin, and the cell size corresponding to 50% reduced grade efficiency is known as the reduced cut size d'_{50} (*Figure 11.6*).

Equation (17), obtained from Equation (10), shows that it is possible to estimate the total efficiency of cell separation when the curves of grade efficiency and feed size distribution are known. Based on Equations (16) and (17), it is possible to obtain a similar result for the reduced total efficiency (Equation 18).

$$E = \int_0^1 G\,dy \tag{17}$$

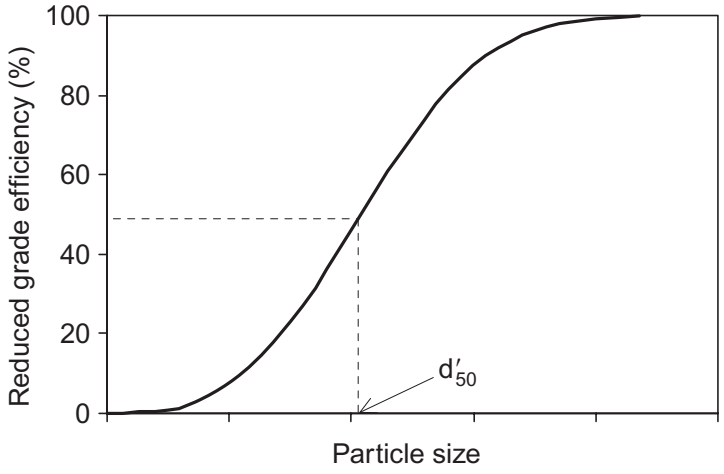


Figure 11.6

Reduced grade efficiency curve and reduced cut size (d'_{50}).

$$E' = \int_0^1 G' dy \tag{18}$$

If a separator, used as a cell retention device in a perfusion bioreactor, is operating with $E < 100\%$, some of the cells are lost in the perfusate. In such a situation, the apparent specific cell growth rate μ_{ap} is given by Equation (19).

$$\mu_{ap} = \frac{1}{X}\frac{dX}{dt} \tag{19}$$

Equation (20) gives a global cell balance in a bioreactor of volume V operating with a perfusion rate D and using a cell retention device that gives a cell retention efficiency E.

$$V\frac{dX}{dt} = \left[\mu V - (1-E)DV\right]X \tag{20}$$

Based on Equations (19) and (20), it is possible to write:

$$\mu_{ap} = \mu - (1-E)D \tag{21}$$

The maximum operational perfusion rate D_{max} is thus just marginally lower than that which results in cell wash-out. Therefore, when working with D_{max}, the cell loss in the perfusate is totally compensated by the cell growth. In this situation, the apparent specific cell growth rate μ_{ap} is zero. Hence, from Equation (21):

$$D_{\max} = \frac{\mu}{1-E} \tag{22}$$

Consequently, the maximum perfusion rate possible that can be used in a perfusion bioreactor is a function of both the specific cell growth rate and the cell retention efficiency.

11.3 Gravity settling

Cells are separated in a gravity settler due to the action of a gravitational field. This type of equipment operates with flow ratios greater than zero. As animal cells have low densities and diameters, their settling velocities are very low. For instance, the values in an aqueous medium at 37°C are in the 1–15 cm h ^{1} range (Castilho and Medronho, 2002). Therefore, the separator settling area has to be large enough to avoid losing cells through the diluted stream, since it would decrease separation efficiency.

There are two main types of gravity settlers, the vertical and the lamellar (*Figure 11.7*). In the latter, a great number of inclined plates, named lamellas, are packed into the settler in a such a way that the plate distance is very small. Gravity drives the cells to settle over the lamella immediately below them. The cells slide down the plate to the bottom of the apparatus, forming a concentrated stream. Theoretically, the total settling area of a lamella settler is the sum of the surface area projection of all lamellas in a horizontal plane. However, according to Svarovsky (2000), due to inefficiencies in the settling process, only around 50% of this area is really effective. Even so, this separator is much more compact than a vertical settler, whose settling area is the cross-sectional area of its cylindrical part.

Gravity settlers are usually designed based on a desired 100% cell separation efficiency. To obtain such efficiency, the terminal settling velocity of the smallest cell ($v_{t,\min}$) should be higher than the ascending velocity of the liquid (u). At the limiting condition ($u = v_{t,\min}$), the minimum required settling area (A_{min}) will be given by Equation (23). The recommended working area for the settler should then be three times this minimum required settling area.

$$A_{\min} = \frac{Q_D}{v_{t,\,\min}} \tag{23}$$

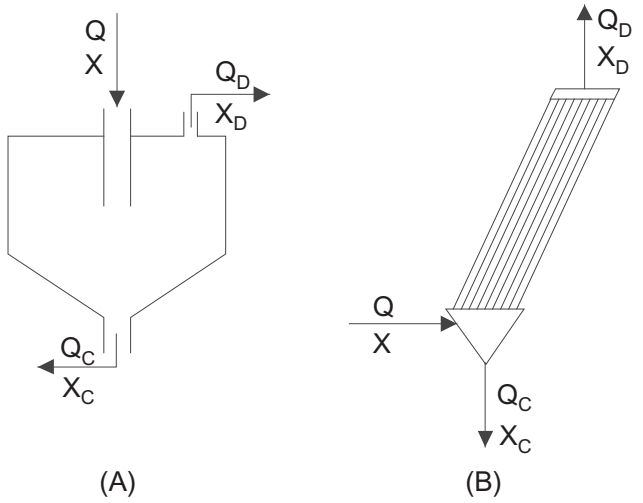


Figure 11.7

Settlers usually employed in cell separations: (A) vertical settler; (B) lamella settler (adapted from Henzler *et al.*, 2003).

Settlers are simple devices and present no moving parts, facilitating operations under aseptic conditions. However, they present operational problems because of the high residence time of the cells in an environment that presents suboptimal conditions of oxygenation and temperature (Woodside *et al*., 1998). They also present difficulties for scale-up since the required settling area is directly proportional to the flow rate to be treated (Equation 23), which increases with bioreactor volume. Lamella settlers are also prone to cell adhesion on the lamella surface. This problem may be diminished by using a special coating on the plates or by installing a vibration system to vibrate the whole settler (Castilho and Medronho, 2002). This implies an increase in the complexity of the device. Despite these considerations, Henzler *et al*. (2003) have successfully employed a lamella settler as the cell retention device in perfusion bioreactor systems. According to the authors, the high cell retention efficiencies obtained with their specially designed lamella settlers allow using perfusion rates as high as 15 d^{-1}.

11.4 Centrifugation

Centrifugation is based on particle settling in a centrifugal field. This centrifugal field is created when applying a centrifugal acceleration to a suspension, through a rotational movement. In a general way, particles more dense than the liquid will attain an outward radial movement and particles less dense will attain an inward radial movement.

The main types of industrial centrifuges used for bioparticle separations are the tubular, the multi-chamber, and the disc centrifuges (*Figure 11.8*). The disc centrifuge may have nozzles to allow continuous exit of the concentrated suspension. The nozzles allow operation in a continuous mode, with $R_f > 0$. Tubular and multi-chamber centrifuges operate with $R_f = 0$.

The settling velocity of bioparticles in centrifuges is given by Equation (1). In this equation, the angular velocity ω is in rad s^{-1}. Equation (24) converts angular velocity expressed in rotations per minute (rpm) to rad s^{-1}.

$$[\omega]_{rad/s} = \frac{\pi}{30}[\omega]_{rpm} \tag{24}$$

When expressing operational conditions of centrifuges, it is advisable to give the centrifugal acceleration ($\omega^2 R$) rather than angular velocity (ω), since the latter does not properly describe the centrifugal field intensity, which is a function of the radius of the centrifuge rotor. Thus, it is usual to use the *g*-factor concept, also known as RCF (relative centrifugal force). This is obtained by dividing the bioparticle settling velocity under a centrifugal field by the bioparticle settling velocity under the gravitational field (Equation 25).

$$g\text{-}factor = \frac{\omega^2 R}{g} \tag{25}$$

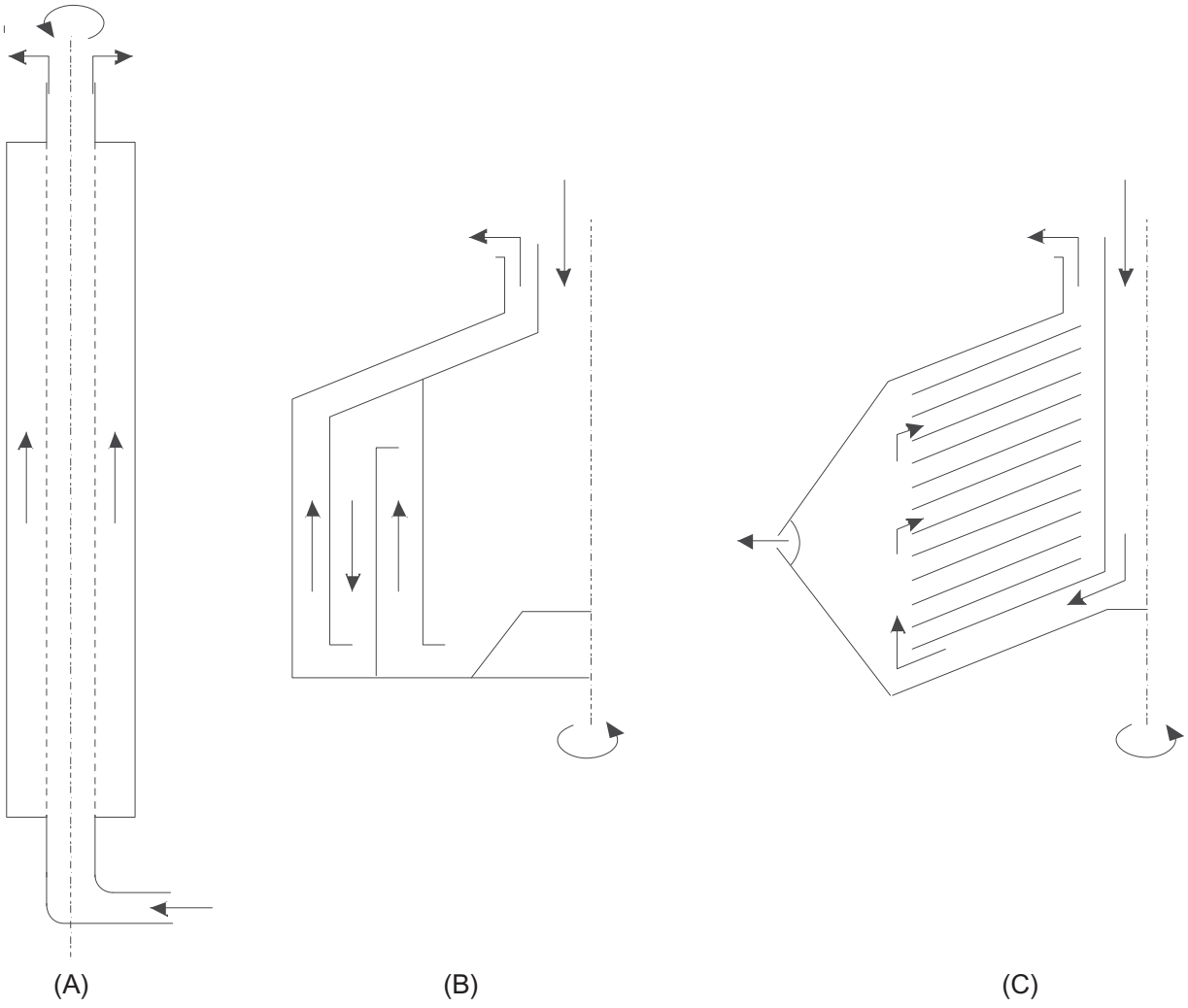


Figure 11.8

Schematic drawing of centrifuges: (A) tubular, (B) multi chamber, (C) disc centrifuge.

Centrifuges give high separation efficiencies for animal cells and may be used either in batch processes (Kempken *et al*., 1995; Tebe *et al*., 1997) or as cell retention devices in bioreactors operating in perfusion mode (Johnson *et al*., 1996; Takamatsu *et al*., 1996). However, centrifuges are complex devices, with moving parts, of relatively high cost, and the cells are submitted to shear stresses that may reach relatively high levels. Bjorling *et al*. (1995) observed cell adhesion problems and clogging of the exit channels when using a disc centrifuge as a cell retention device in a perfusion culture.

In an attempt to eliminate some of these problems, a new centrifuge (Centritech®) was specially designed for mammalian cell separation (Johnson *et al*., 1996). This centrifuge operates with $R_f > 0$ and generates a centrifugal field intensity up to 320 *g*. It operates in a closed system and contains a presterilized and disposable plastic insert, which eliminates the

necessity for CIP (cleaning in place) and SIP (sterilization in place). The available models may be employed for cell separation of batch processes dealing with volumes from 5 to 600 L, or in perfusion processes coupled to 5–3000 L bioreactors (Centritech®, 2006).

11.5 Hydrocyclones

Hydrocyclones are very simple devices and always operate with a flow ratio $R_f > 0$. They may be easily designed to give a desired separation efficiency (Castilho and Medronho, 2000), and their performance may also be easily predicted (Coelho and Medronho, 2001). In the last few years, it has been shown either theoretically or experimentally that hydrocyclones may be used in animal cell separations (Luebberstedt *et al.*, 2000; Medronho *et al.*, 2005; Elsayed *et al.*, 2006; Pinto *et al.*, 2007) aimed mainly at mammalian cell retention in perfusion bioreactors (Jockwer *et al.*, 2001; Elsayed *et al.*, 2005).

Hydrocyclones are similar to centrifuges in that they use cell settling in a centrifugal field as the principle of separation. A hydrocyclone (*Figure 11.9*) consists of a conical section joined to a cylindrical portion,

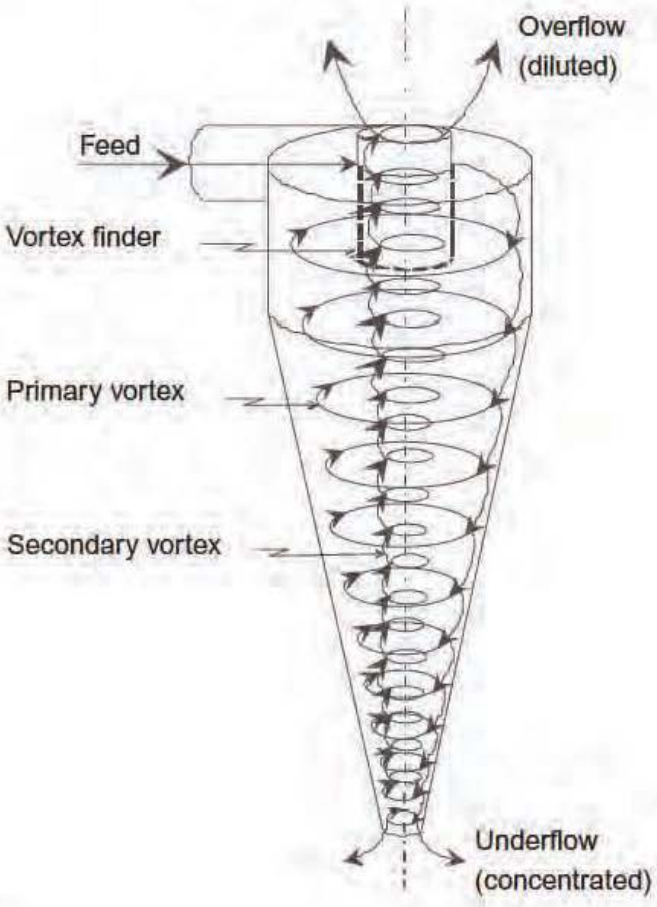


Figure 11.9

Perspective view of a hydrocyclone showing the liquid flow path.

which is fitted with a tangential inlet and closed by an end plate with an axially mounted overflow pipe, also called a vortex finder. The end of the cone terminates in a circular apex opening, called an underflow orifice.

The liquid flow path inside a conventional hydrocyclone is shown in *Figure 11.9*. The culture medium is pumped through the feed pipe and upon entry into the device, acquires a downward rotational movement (primary vortex) trying to leave the device through the underflow orifice. As this opening is not large enough, only part of the liquid is able to escape – carrying with it the larger cells, forming the concentrated stream. The fraction of liquid that is not able to leave the hydrocyclone through the apex opening reverses its movement, creating an upward rotational movement (secondary vortex), which finally leaves the device through the overflow pipe (diluted stream) carrying the smallest particles, including small cells and debris.

In spite of using the same separation principle, the main difference between a centrifuge and a hydrocyclone is that the latter does not have moving parts. Therefore, hydrocyclones can cope well with the aseptic operations required by the biopharmaceutical industry. Apart from that, when separating cells, they do not require any maintenance during their working life. This means that they can be operated during very long periods as cell retention devices in perfusion processes.

Pinto *et al*. (2007) reported that a hydrocyclone specially designed for animal cell separations (Deckwer *et al*., 2005) was able to produce total separation efficiencies above 97% for CHO cells. The possible effects of the device on the cells were evaluated by different techniques, including the traditional viability measurement method based on trypan blue exclusion. Measurements of lactate dehydrogenase activity in the culture medium to verify if this intracellular enzyme had been released to the extracellular medium due to cell lysis, indicated that the hydrocyclone effect on cell viability was negligible. The concentration of apoptotic cells monitored by fluorescence microscopy showed that passing the cells through the hydrocyclone did not induce cell death by apoptosis. Moreover, the cell viability at the concentrated stream (97%) and at the diluted stream (82%) indicated that the hydrocyclone could selectively retain viable cells. This is highly desirable when conducting a perfusion process. This hydrocyclone specially designed for animal cell separation was also evaluated on a pilot scale by Elsayed *et al*. (2005). These authors, when using the hydrocyclone coupled to a 30 L bioreactor operating in perfusion mode with HeLa cells, found cell viabilities always above 93%. The same hydrocyclone was also tested on an industrial scale by the authors of this chapter. In these tests, the bioreactor had a volume of 300 L and operated with NS0 cells producing a monoclonal antibody (mAb) (unpublished data). The results confirmed that the hydrocyclone was capable of promoting cell retention in a perfusion system, which could be operated at high cell viability levels (*Figure 11.10A*), and that the separation efficiency for viable cells was higher than that for dead cells (*Figure 11.10B*). This confirmed the capability of a hydrocyclone to selectively retain viable cells in the bioreactor.

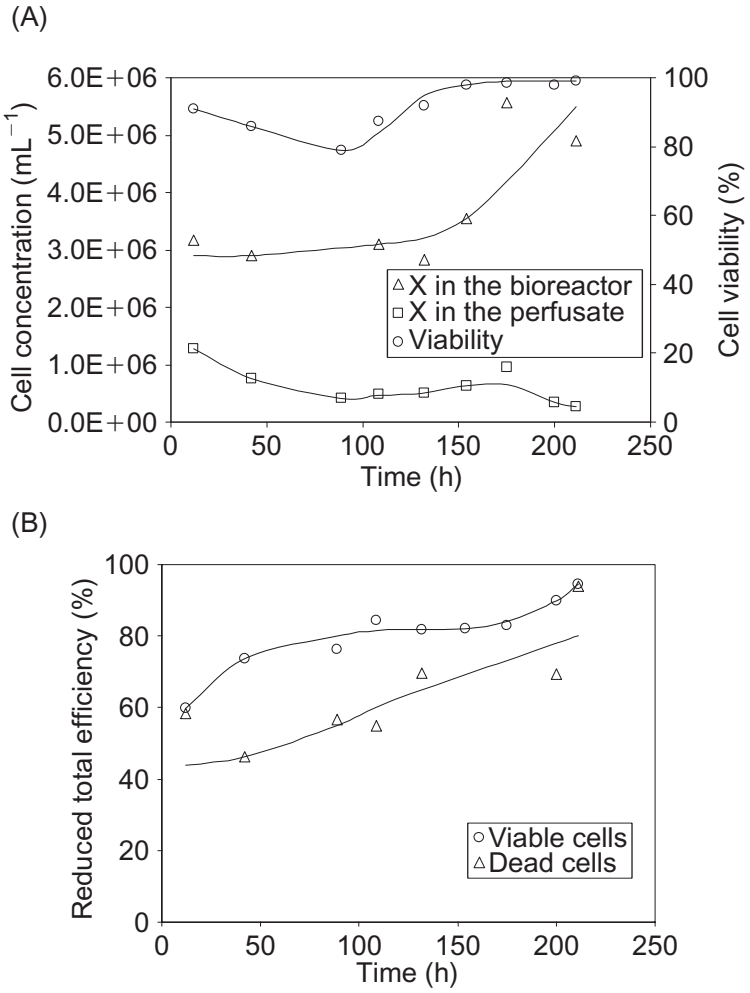


Figure 11.10

Results for a 300 L bioreactor operating in perfusion mode using a specially designed hydrocyclone as a cell retention device.

11.6 Filtration

Filtration is a unit operation commonly employed nowadays in biotechnological processes. In this unit operation, a filter medium acts as a physical barrier to particles larger than its pores. Traditional filtration devices such as filter presses and rotary vacuum drum filters have so far found no application for the separation of animal cells. Nevertheless, membrane filters are commonly employed, as well as some alternative filter designs such as spin-filters. In the next sections, the most common types of filters used for animal cell separation will be discussed.

11.6.1 Tangential flow filtration with membranes

Tangential filtration is distinguished from conventional filtration, also known as dead-end filtration, by the fact that the main flow is tangential to the membrane surface (*Figure 11.11*). In dead-end filtration a fast drop in the permeate flux is observed due to cake formation over the filter

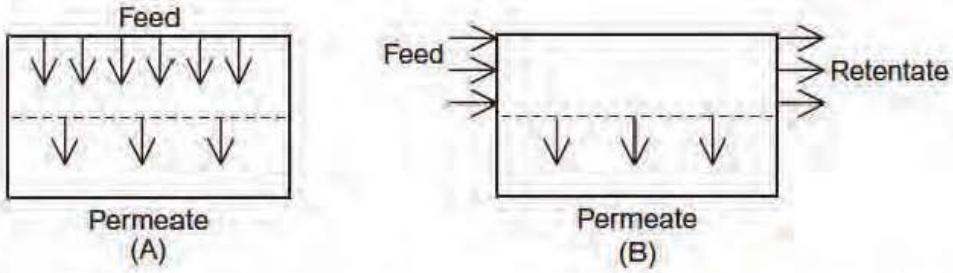


Figure 11.11

Schematic representation of dead end filtration (A) and tangential filtration (B).

medium, but in tangential filtrations this drop in the permeate flux is less accentuated. Therefore, in systems with adequate hydrodynamic conditions, tangential filters may operate continuously for relatively long periods, under stable flux conditions.

In tangential filtration, membranes are used as filter media. Membranes are defined as barriers of reduced thickness, across which physical and/or chemical gradients are established to facilitate the preferential migration of one or more components from a given mixture, promoting their separation (Klein, 1991). They are usually made of polymers or inorganic materials, such as ceramic or sintered steel. In the biopharmaceutical industry, membranes find various applications, such as production of water for injection (WFI), sterilization of culture media, buffer solutions and gases, separation of cells and cell debris, and purification and concentration of proteins.

Microporous membranes with nominal pore sizes in the 0.1–10 μm range are usually employed in animal cell separation. Such membranes are able to retain the cells, but unable to retain solutes of low or high molar mass (*Figure 11.12*). Microfiltration uses a pressure drop, usually in the 0.5–2 bar range, as the driving force to promote separation (Nobrega *et al.*, 2005).

Two main types of membrane modules are normally employed for animal cell separation: the plate-and-frame and the hollow-fiber modules. The latter type has as an important feature, a high packing density, which results in a high permeation area for a compact module. Both module types may be used in cell separation in batch processes, and also in perfusion processes. The main characteristics of these two module types are described in *Table 11.1*.

Tangential filtration of animal cell suspensions presents several advantages, such as easy scale-up, simple operation, and a permeate totally free



Figure 11.12

Schematic representation of a microfiltration process. The applied pressure drop (ΔP) is usually in the range of 0.5 2 bar.

Table 11.1 Comparison between hollow-fiber and plate-and-frame modules

Characteristics	Hollow fiber	Plate and frame
Internal diameter (μm)	40 500	
External diameter (μm)	80 800	
Membrane area/module volume (m^2 m^{-3})	5000 10000	100 600
Maximum operational pressure (bar)	100 (shell), 15 (fibers)	80

Adapted from Nobrega *et al.* (2005) and Rautenbach (1997).

from cells, which allows, for instance, direct application of the permeate to chromatography columns. However, it also has some serious limitations related to concentration, polarization and membrane fouling. The first phenomenon is a reversible one and occurs due to the formation of a concentration gradient of solutes on the membrane surface, at the beginning of the filtration process. Membrane fouling, however, is generally an irreversible phenomenon. It occurs due to membrane modifications caused by components present in the feed stream. These modifications arise mainly from adsorption of some molecules onto the membrane, deposition of suspended material onto the membrane surface and pore clogging by suspended particles (Nobrega *et al.*, 2005). These phenomena cause a drop in permeate flux and an increase in solute rejection with filtration time (Cheryan, 1998).

Tangential filtration was developed in an attempt to reduce the drop in permeate flux, by the generation of high shear stresses on the membrane surface, which avoids material deposition and reduces progressive membrane clogging. However, when the suspension to be filtered contains animal cells, the operating flow rate and, consequently, the shear stress generated need to be low because of the fragility of the cells. Different strategies have been proposed in the literature to reduce the progressive drop in permeate flux. These strategies include periodic back-flushing, use of a pulsating feed flow, and coating the membrane with polyethylene glycol to avoid cell and protein adhesion (Smith *et al.*, 1991; Zhang *et al.*, 1993). Even so, there are few published reports of perfusion processes employing tangential filtration for long periods ($>$ 20 days).

On the other hand, for cell separations at the end of a batch process, maintaining a high cell viability is a less critical issue than in perfusion processes, so that higher shear stress levels are acceptable. Moreover, the filtration periods are never longer than a few hours. Thus, in batch processes the advantages of using tangential filtration are greater than its disadvantages.

11.6.2 Dynamic filters

The removal of particles accumulated on a filter medium is strongly influenced by the shear rates generated on its surface (Serra *et al.*, 1999; Silva *et al.*, 2000). Therefore, most membrane modules try to avoid clogging by maximizing the shear rates (Belfort *et al.*, 1994). However, this is not always possible in the case of animal cells, due to their shear

sensitivity. Furthermore, in conventional tangential flow filtration, feed flow rate and shear rate levels are intrinsically coupled.

Dynamic filtration modules present a relative movement between the membrane and the module, or between the membrane and a rotor. Thus, it is possible to adjust the shear stress independently of the feed flow rate and of the transmembrane pressure drop.

Dynamic filtration modules are basically of two types: rotating disc filter (RDF) and vortex flow filter (VFF). In the latter, the filtration module has a cylindrical shape and has a rotating concentric cylindrical mesh in its interior. The rotational movement of the internal cylinder generates a Taylor-Couette flow in the annular gap (Roth *et al*., 1997), creating Taylor vortices that minimize concentration polarization and mesh fouling. Continuous perfusion processes based on this type of filter and operating continuously for up to 100 days have been reported (Mercille *et al*., 1994).

In RDFs, a disc-shaped rotor is located inside the module. By adjusting the rotational velocity of the rotor, it is possible to carefully control the shear stress levels according to the sensitivity of the cells being used. Therefore, RDFs have been successfully used to separate animal cells, mainly in batch processes (Kempken *et al*., 1997; Vogel and Kroner, 1999).

Castilho and Anspach (2003) have developed an RDF (*Figure 11.13*) specially designed for mammalian cell separation. These authors employed computational fluid dynamics (CFD) to optimize the filter geometry and also to study the influence of rotor angular velocity on the shear stress generated in the device. This filter was successfully used as a cell retention device for perfusion cultivation of CHO cells producing a mAb. It was also used in an integrated cell separation/product purification process, using affinity membranes containing protein A as ligand for antibody adsorption (Castilho *et al*., 2002).

11.6.3 Spin-filters

Spin-filters are cell retention devices used almost exclusively in perfusion processes with animal cells. It is common to find reports in the literature referring to internal and external spin-filters. However, in this chapter the latter have been classified as VFFs and as such were described in Section

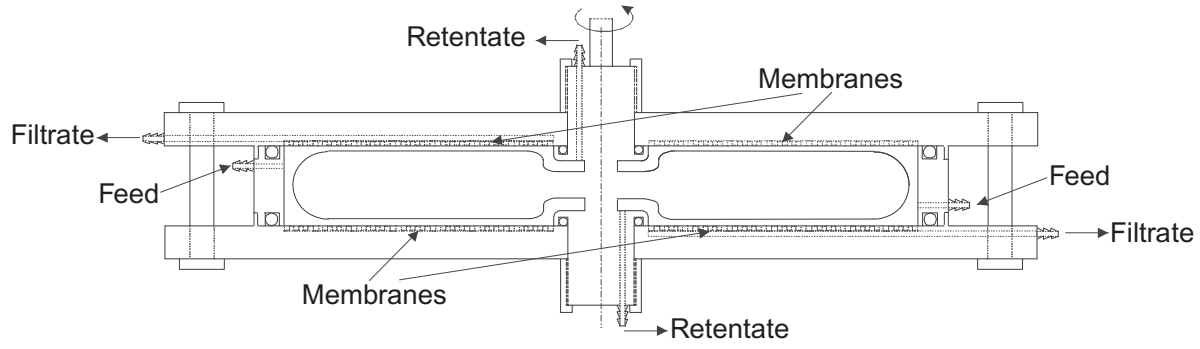


Figure 11.13

Rotating disc filter developed by Castilho and Anpach (2003).

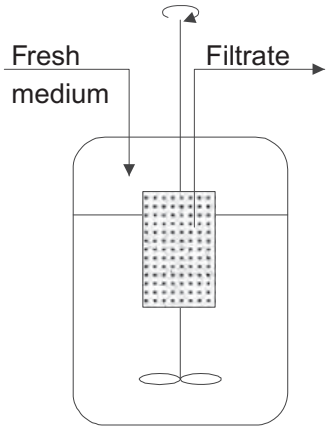


Figure 11.14

Schematic representation of a spin filter fitted on the impeller shaft of a bioreactor.

11.6.2. The internal spin-filter consists of a cylinder with porous walls (normally a mesh), which rotates inside a bioreactor. It may be driven by an independent motor or may be fitted on the impeller shaft (*Figure 11.14*). Culture medium is continuously removed through the mesh, under vacuum, while fresh medium is added at the same volumetric rate to the bioreactor.

Mesh fouling is the main problem associated with the operation of a spin-filter. As it is located inside the bioreactor vessel, it is impossible to exchange the filter under sterile conditions once it has clogged. When this happens, the culture must be terminated.

Apart from fouling, cell retention efficiency is another important factor to be considered. An efficiency within a range of 63–99% is normally achieved when working with spin-filters (Castilho and Medronho, 2002). In reality, the mechanism of cell retention is not yet well understood. That is why spin-filter design and optimization of operational conditions are still performed empirically. Cells are retained inside the bioreactor as a function of several forces. The main forces involved are the centrifugal and lift forces, arising from spin-filter rotation, and the drag force due to culture medium removal through the spin-filter. However, the interactions between those forces are not well understood. Thus, there are some reports of high retention efficiencies (99%) being achieved using spin-filters with a large mesh opening (127 μm) (Avgerinos *et al*., 1990), while other authors found smaller efficiencies (75–95%) when using a mesh opening of only 20 μm (Iding *et al*., 2000). These discrepancies show the necessity of a better understanding of the fluid dynamics involved in cell retention using spin-filters. In this context, CFD may be a valuable tool to facilitate this understanding (Castilho and Medronho, 2002; Figueredo-Cardero *et al*., in press).

11.7 Ultrasonic separation

Ultrasonic separators, also known as acoustic cell filters, use a plane standing-wave to retain cells in a bioreactor. Kilburn *et al*. (1989) were the

first authors to report this possibility. Since then, many reports have been published about their use at different scales and with different cells (Shirgaonkar *et al*., 2004).

The ultrasonic separation of cells occurs due to the forces that are generated in a plane standing-wave. These forces are the result of interactions between the cells and the fluid, and their magnitude is a function of density and compressibility differences between the cells and the fluid. A plane standing-wave occurs as a result of the interference between two waves of the same wavelength and amplitude traveling in opposite directions. In such a situation, the cells are retained in pressure nodes (*Figure 11.15*), forming cell aggregates. The device is operated in cycles, and its periodic shut-down allows the cell aggregates to settle back towards the bioreactor.

This type of separator has been used basically for cell retention in perfusion processes. It operates directly coupled to the top of a bioreactor. To maximize retention efficiency and cell viability when using it, different operational parameters should be optimized, such as cycle duration, perfusion and recirculation flow rates, acoustic force intensity, and the separator backwash frequency (Shirgaonkar *et al*., 2004).

Gorenflo *et al*. (2005) employed an experimental design methodology for optimizing the operational conditions of an acoustic filter with a 10 L d^{-1} capacity. They observed a high operational stability of the device, allowing for its use in long-term perfusion runs. Dalm *et al*. (2005) also investigated the long-term stability of a 200 L d^{-1} acoustic filter. The authors verified that the separator presented a simple and stable operation throughout 75 days of perfusion cultivation of a hybridoma.

Several different cell lines have already been investigated in perfusion cultures using the acoustic cell filter. For instance, Shirgaonkar *et al*. (2004) used NS0, HEK-293, SP2-derived hybridoma, and insect cells in different serum-supplemented and serum-free media at different perfusion rates and acoustic chamber volumes. According to these authors, an adequate operation of the filter depends on optimization of operational

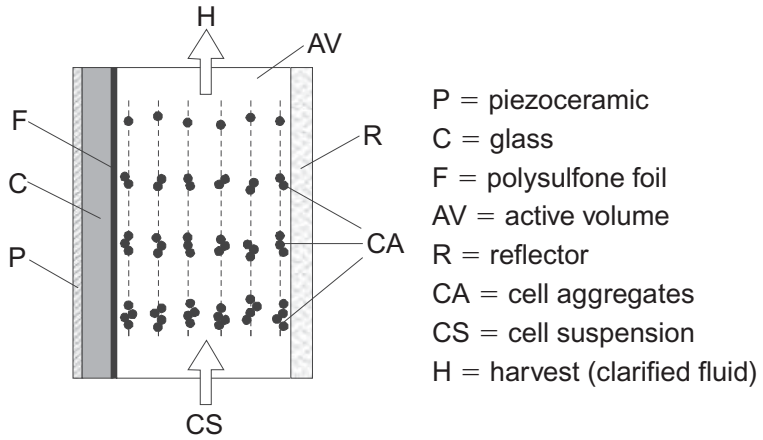


Figure 11.15

Schematic drawing of the acoustic cell filter employed by Groeschl *et al*. (1998). The pressure nodes are represented by dashed lines.

conditions and strategies. For instance, when working at high cell densities, nutrient and oxygen depletion may occur during the period of time the cells remain inside the separator. Another concern is the possible clogging of the bottom part of the acoustic filter. Those authors developed an efficient recirculation and backwashing technique that avoids clogging. However, these techniques increase the complexity level of the acoustic filter operation.

References

Avgerinos GC, Drapeau D, Socolow JS, Mao J, Hsiao K, Broeze RJ (1990), Spin filter perfusion system for high density cell culture: production of recombinant urinary type plasminogen activator in CHO cells, Bio/Technol. 8:54 58.

Belfort G, Davis RH, Zydney AL (1994), The behavior of suspensions and macro molecular solutions in cross flow microfiltration, J. Membr. Sci. 96:1 58.

Björling T, Dudel U, Fenge C (1995), Evaluation of a cell separator in large scale perfusion culture, In: Beuvery EC, Griffiths JB, Zeijlemaker WP (Eds), Animal Cell Technology: Developments Towards the 21st Century, Kluwer Academic, Dordrecht, pp. 671 673.

Castilho LR, Anspach FB (2003), CFD aided design of a dynamic filter for mammalian cell separation, Biotechnol. Bioeng. 83:514 524.

Castilho LR, Medronho RA (2000), A simple procedure for design and performance prediction of Bradley and Rietema hydrocyclones, Minerals Eng. 13:183 191.

Castilho LR, Medronho RA (2002), Cell retention devices for suspended cell perfusion cultures, Adv. Biochem. Eng. Biotechnol. 74:129 169.

Castilho LR, Anspach FB, Deckwer W D (2002), An integrated process for mamma lian cell perfusion cultivation and product purification using a novel dynamic filter, Biotechnol. Progr. 18:776 781.

Centritech® (2006), Product information. Available at: http://www.pneumaticscale. com/content/menus/psc/centritech centrifuge.aspx (accessed March 2007).

Cheryan M (1998), Ultrafiltration and Microfiltration Handbook, Technomic Publish ing, Lancaster.

Coelho MAZ, Medronho RA (2001), A model for performance prediction of hydro cyclones, Chem. Eng. J. 84:7 14.

Dalm MCF, Jansen M, Keijzer, TMP, Grunsven WMJ, Oudshoorn A, Tramper J, Martens DE (2005), Stable hybridoma cultivation in a pilot scale acoustic perfu sion system: long term process performance and effect of recirculation rate, Biotechnol. Bioeng. 91:894 900.

Deckwer W D, Anspach FB, Medronho RA, Luebberstedt M (2005), Method for separating viable cells from cell suspension, Patent US 6878545 B2.

Elsayed A, Piehl G W, Nothnagel J, Medronho RA, Deckwer WD, Wagner R (2005), Use of hydrocyclone as an efficient tool for cell retention in perfusion cultures, In: Gòdia F, Fussenegger M (Eds), Animal Cell Technology Meets Genomics Proceedings of the 18th ESACT Meeting (European Society for Animal Cell Technology), Kluwer Academic Publishers, Dordrecht, pp. 679 682.

Elsayed A, Medronho RA, Wagner R, Deckwer, W D (2006), Use of hydrocyclones for mammalian cell retention I. Separation efficiency and cell viability, Eng. Life Sci. 6:347 354.

Figueredo Cardero A, Chico E, Castilho LR, Medronho RA (in press), CFD study of the fluid and particle dynamics in a spin filter perfusion bioreactor, In: Hauser H (Ed.), Proceedings of the 20th ESACT Meeting (European Society for Animal Cell Technology), Springer, Dordrecht.

Gorenflo VM, Ritter JB, Aeschliman DS, Drouin H, Bowen BD, Piret JM (2005),

Characterization and optimization of acoustic filter performance by experimental design methodology, Biotechnol. Bioeng. 90:746 753.
Gröschl M, Burger W, Handl B (1998), Ultrasonic separation of suspended particles Part III: Application in biotechnology, Acustica 84:815 822.
Henzler H, Kauling J, Schmitt F, Beckers E, Boedeker B, Von Hugo H, Konstantinov K, Naveh D (2003), Continuous high cell density fermentation unit for cultivating suspended animal or plant cell lines uses sedimentation separator of specific surface area, Patent WO2003020919 A2.
Iding K, Lütkemeyer D, Fraune E, Gerlach K, Lehmann J (2000), Influence of alterations in culture condition and changes in perfusion parameters on the retention performance of a 20 μm spinfilter during a perfusion cultivation of a recombinant CHO cell line in pilot scale, Cytotechnology 34:141 150.
Jockwer A, Medronho RA, Wagner R, Anspach FB, Deckwer WD (2001), Use of hydrocyclones for mammalian cell retention in perfusion bioreactors, In: Olsson EL, Chatzissavidou N, Lüllau E (Eds), Animal Cell Technology: From Target to Market Proceedings of the 17th ESACT Meeting (European Society for Animal Cell Technology), Kluwer Academic Publishing, Dordrecht, pp. 301 305.
Johnson M, Lantier S, Massie B, Lefebvre G, Kamen AA (1996), Use of the Centritech® lab centrifuge for perfusion culture of hybridoma cells in protein free medium, Biotechnol. Progr. 12:855 864.
Kempken R, Preissmann A, Berthold W (1995), Assessment of a disc stack centrifuge for use in mammalian cell separation, Biotechnol. Bioeng. 46:132 138.
Kempken R, Rechtsteiner H, Schäfer J, Katz U, Dick O, Weidemeier R, Sellick I (1997), Dynamic membrane filtration in mammalian cell culture harvest, In: Carrondo MJT (Ed.), Animal Cell Technology, Kluwer, Dordrecht, pp. 379 382.
Kilburn DG, Clarke DJ, Coakley WT, Bardsley DW (1989), Enhanced sedimentation of mammalian cells following acoustic aggregation, Biotechnol. Bioeng. 34: 559 562.
Klein E (1991), Affinity Membranes Their Chemistry and Performance in Adsorp tive Separation Processes, John Wiley and Sons, New York.
Luebberstedt M (2000), Separation tierischer Zellkulturen durch Einsatz von Hydro zyklonen, Diplomarbeit des Studienganges Biotechnologie der Technische Fach hochschule Berlin, Berlin.
Luebberstedt M, Medronho RA, Anspach FB, Deckwer W D (2000), Abtrennung tierischer Zellen mit Hydrozyklonen, Chem. Ing. Tech. 72:1089 1090.
Medronho RA (2003), Solid liquid separation, In: Mattiasson B, Hatti Kaul R (Eds) Isolation and Purification of Proteins, Marcel Dekker, New York, pp. 131 190.
Medronho RA, Schütze J, Deckwer W D (2005), Numerical simulation of hydrocy clones for cell separation, Latin Am. Appl. Res. 35:1 8.
Mercille S, Johnson M, Lemieux R, Massie B (1994), Filtration based perfusion of hybridoma cultures in protein free medium reduction of membrane fouling by medium supplementation with DNAse I, Biotechnol. Bioeng. 43:833 846.
Nobrega R, Borges CP, Habert AC (2005), Processos de separação com membranas, In: Pessoa A Jr, Kilikian BV (Eds), Purificação de Produtos Biotecnológicos, Editora Manole, Barueri/SP, pp. 37 88 (in Portuguese).
Pinto RCV, Medronho RA, Castilho LR (2007), The effects of cell separation with hydrocyclones on the viability of CHO cells, In: Smith R (Ed.), Cell Technology for Cell Products Proceedings of the 19th Meeting of ESACT (European Society for Animal Cell Technology), Springer, Dordrecht, pp. 745 748.
Rautenbach R (1997), Membranverfahren Grundlagen der Modul und Anlagenaus legung, Springer Verlag, Berlin, pp. 60 77 (in German).
Roth G, Smith CE, Schoofs GM, Montgomery TJ, Ayala JL, Horwitz JI (1997), Using an external vortex flow filtration device for perfusion cell culture, BioPharm. 10:30 34.

Schulz C (1996), Einsatz eines Scherfilters zur Separation von Tierischen Zellen aus kontinuierlicher Kultivierung, Diplomarbeit des Studienganges Biotechnologie der Technischen Universität Carolo Wihelmina, Braunschweig (in German).
Serra CA, Wiesner MR, Laine JM (1999), Rotating membrane disk filters: design evaluation using computational fluid dynamics, Chem. Eng. J. 72:1 5.
Shirgaonkar IZ, Lanthier S, Kamen A (2004), Acoustic cell filter: a proven cell retention technology for perfusion of animal cell cultures, Biotechnol. Adv. 22:433 444.
Silva CM, Reeve DW, Husain H, Rabie HR, Woodhouse KA (2000), Model for flux prediction in high shear microfiltration systems, J. Membr. Sci. 173:87 98.
Smith CG, Guillaume JM, Greenfield PF, Randerson DH (1991), Experience in scale up of homogeneous perfusion culture for hybridomas, Bioproc. Eng. 6:213 219.
Svarovsky L (2000), Gravity clarification and thickening, In: Svarovsky L (ed), Solid Liquid Separation, Butterworth Heinemann, Oxford.
Takamatsu H, Hamamoto K, Ishimaru K, Yokoyama S, Tokashiki M (1996), Large scale perfusion culture process for suspended mammalian cells that uses a centrifuge with multiple settling zones, Appl. Microbiol. Biotechnol. 45:454 457.
Tebe H, Lütkemeyer D, Gudermann F, Heidemann R, Lehmann J (1997), Lysis free separation of hybridoma cells by continuous disc stack centrifugation, Cytotech nology 22:119 127.
Vogel JH, Kroner KH (1999), Controlled shear filtration: a novel technique for animal cell separation, Biotechnol. Bioeng. 63:663 674.
Woodside SM, Bowen BD, Piret JM (1998), Mammalian cell retention devices for stirred perfusion systems, Cytotechnology 28:163 175.
Zhang S, Handa Corrigan A, Spier RE (1993), A comparison of oxygenation methods for high density perfusion cultures of animal cells, Biotechnol. Bioeng. 41: 685 692.

Product purification processes

12

Ângela Maria Moraes, Leda dos Reis Castilho, and Sonia Maria Alves Bueno

12.1 Introduction

The development of methods and techniques for the separation and purification of biological macromolecules such as proteins has been a prerequisite for advances in bioscience and biotechnology during the last five decades.

The recovery and purification of a bioproduct is carried out to isolate it from its production system (cell culture, plant or animal tissues), and to obtain the required purity and formulation. Before establishing a strategy for recovery and purification, it is essential to collect all available information related to the protein and to the medium where it is found. Usually, not only the theoretical information, but also preliminary experiments are needed. It is also noted that the feasibility of a process on a laboratory scale does not guarantee its feasibility on an industrial scale.

With the use of high performance materials and automated instruments, protein separation is becoming a more controllable process. However, some problems persist even with the use of sophisticated instruments. Many difficulties are still found in determining the optimal extraction and purification conditions, as well as in selecting suitable methods for detecting the protein and quantifying its biological activity.

A general scheme of the recovery and purification process for a bioproduct is shown in *Figure 12.1*. Despite the lack of a universal strategy, some general directions (listed below) should be followed when establishing the purification protocol:

(i) choose separation methods based on different physical principles;
(ii) choose methods based on physical properties related to the greatest differences between products and impurities;
(iii) first remove contaminants that are most dissimilar to the product and/or those that are most abundant;
(iv) assign the most demanding process to the end of the sequence.

12.2 Basic considerations

Before developing an isolation and purification process for a protein, some considerations should be made regarding the final application of the product, the most suitable source material and the available information

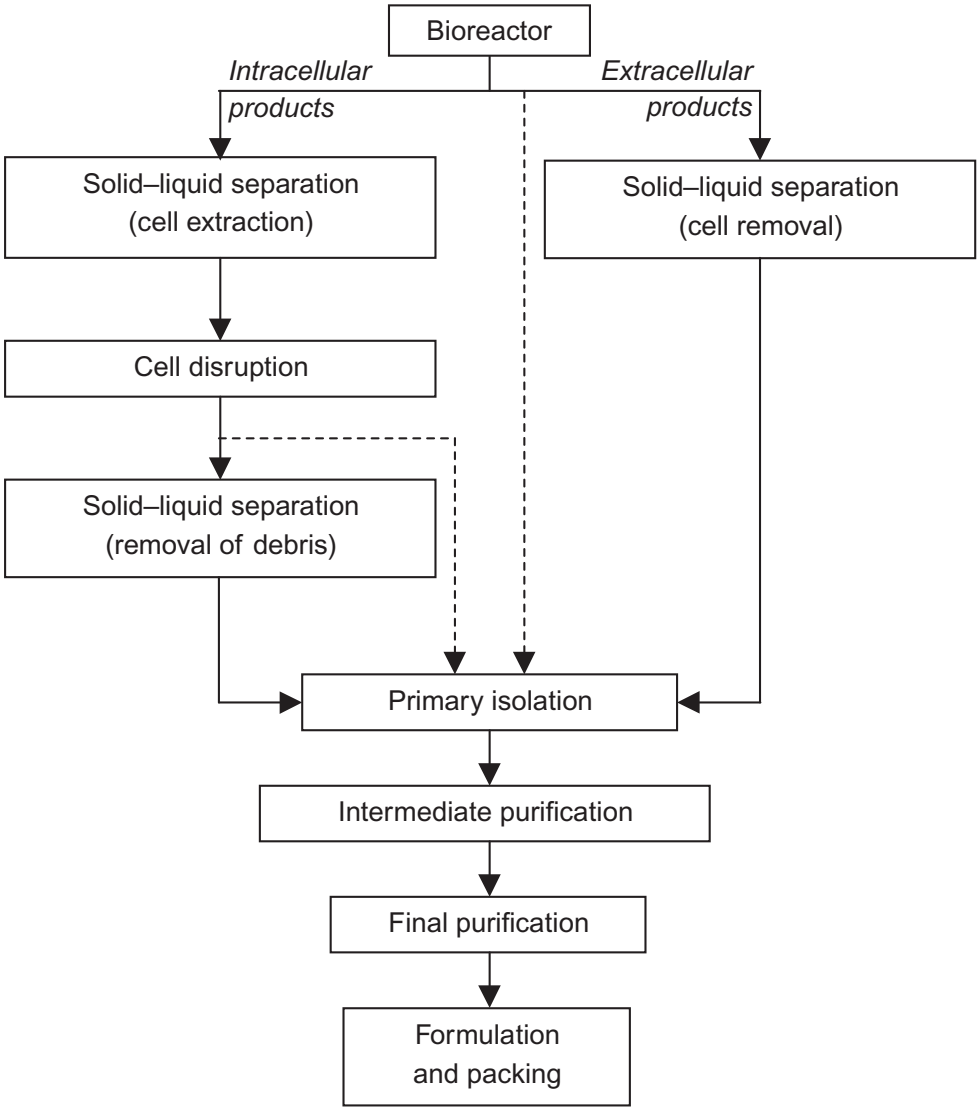


Figure 12.1

General scheme of a bioproduct recovery and purification process. The dotted arrows indicate integrated processes (see Section 12.4.6).

about the product, including existing data on manipulation and quantification (Harris and Angal, 1989).

From these preliminary data, the objectives and performance criteria of the purification sequence can be defined and, thus, the fundamental basis for designing the most suitable strategy is established.

12.2.1 Final application of product

Usually protein purification represents the route for obtaining the product in a pure form, suitable for different applications such as: activity and structural studies, studies of the structure–function relationship of the protein, and its clinical or industrial applications. The purpose of the application determines the required purity of the protein, the acceptable

loss of activity, and also, the acceptable time and cost of the purification process.

Therefore, for activity-related studies, relatively small amounts of protein are needed and high purity is certainly not crucial, provided that the activity-interfering species are removed. Cost in this case has relatively low relevance, but speed is important to minimize activity loss.

Structural studies, on the other hand, require large amounts of highly purified protein. Cost and time are of secondary importance, except in studies regarding structure–function relationship, for which maintenance of biological activity is important and thus, the purification speed is important.

Purification cost and time are most relevant for industrial scale production. The required amounts and purity levels are determined based on the final application of the protein. For example, for therapeutic applications high purity is crucial, but the required amounts are relatively small.

The amount of purified protein obtained depends not only on the amount of the raw material used, but also on the yield of the process. At every stage of the purification process, some product loss occurs. Therefore, to maximize the yield, a minimum number of stages should be used. However, decreasing the number of stages can affect the final purity of the protein. Some purification methods give a higher yield than others and thus, a systematic selection should be made that is aimed at maximizing the yield at each stage, but also insuring the required final purity is obtained.

The purity of the target protein can be expressed in terms of the percentage of total protein, although other types of contaminants can also be important. For enzymatic studies, 80–90% of purity is usually suitable, not taking into account the activity-interfering contaminants, which should be removed. For structural studies, the required purity should be equal to or higher than 95%. For therapeutic applications, any type of contaminants should be removed and purity higher than 99.9% is often required.

Additional purification steps lead not only to additional yield losses, but also to an increase in processing time and cost. For research purposes, the process scales are usually small and the cost of an additional step may not be important. On the other hand, at an industrial scale, the introduction of an additional step may make a purification process uneconomic. Removal of residual contaminants is, in most of the cases, significantly more difficult than the earlier purification steps and involves several additional steps, reducing the final yield and increasing the processing cost and time.

In most cases, except for structural studies such as sequencing, the purified protein should be as native and as active as possible. Attempts to minimize denaturation and proteolysis should be made, avoiding typically harsh conditions.

12.2.2 Selection of the protein source

As discussed in the initial chapters of this book, genetically modified animal cells are commonly used to obtain proteins for therapeutic, prophylactic, or diagnostic applications. The problem is that obtaining

bioproducts from animal cell cultures is limited by the low protein concentration observed in these systems, making the purification process difficult and expensive. On the other hand, animal cells can secrete recombinant proteins into the extracellular medium, allowing easier and cheaper purification, as compared with heterologous proteins found as insoluble intracellular aggregates in bacteria.

To facilitate the purification, the target biomolecule can be modified by, for instance, coupling to it a peptide segment that can be recognized and captured by an affinity ligand attached to a chromatographic matrix. After purification, the inserted fragment can be removed by chemical or enzymatic cleavage (Scheich *et al*., 2003).

The culture medium containing the bioproduct should be processed as soon as possible, to avoid the possibility of degradation, for example, by oxidation or the action of proteases. When immediate processing of extracellular products is not possible, it is recommended to remove the cells and freeze the material, provided the bioproduct is stable to freezing.

12.2.3 Protein properties and manipulation

Knowledge of the physical and chemical properties as well as the cellular location of the target protein helps in designing the separation process. Whether the protein is intra- or extracellular, soluble or insoluble in the cytoplasm, bound to the cell membrane or located within the organelles, will affect the selection of the separation method and the type of buffering system. Proteins bound to the cell membrane require detergents or organic solvents for their solubilization.

Apart from the general problems of solution handling, protein solutions should be handled to prevent denaturation, aggregation, and degradation. Adequate cleaning and sanitization of all equipment is essential in the manipulation of such biomolecules.

12.3 Cell disruption

Bioproducts are usually secreted from animal cells in culture, and can be purified after cell removal by solid–liquid separation techniques (see Chapter 11). However, the product can sometimes be found within the cell and this requires its extraction from the cellular mass, which contains numerous molecular species that can have high viscosity and proteolytic activity, which increases the difficulty of sample handling.

The extraction of an intracellular protein usually involves a compromise between recovery and purity. Optimization of the extraction conditions should maximize the release of the target protein, while minimizing the contaminants, which may be difficult to remove. For this, it is important to determine the conditions under which denaturation or degradation occurs.

There are several available methods for disrupting cells or tissues. The operational conditions can be optimized through the systematic variation of parameters such as medium composition, time, temperature, stirring rate, and size and shape of the blades. Selection of a suitable procedure

demands preliminary tests in which aliquots of the sample are taken at different time intervals and analyzed for product concentration and activity.

The recovery of intracellular proteins involves distinct cell disruption procedures, depending on the cell characteristics. For the processing of animal cells, which do not have a cellular wall, mild and moderate techniques are commonly used. Mild techniques include cell lysis by enzymatic digestion, chemical solubilization or autolysis and the use of manual homogenizers and grinders, whereas the moderate techniques involve blade homogenizers and abrasive grinding.

When the protein is present within a cellular organelle, these methods can still be suitable. However, they may be preceded by isolation of the organelles. Sometimes, the protein has low solubility in the extraction medium, and produces a particulate system requiring specific techniques. These proteins can be extracted through thermal, chemical, or enzymatic treatments, and in some cases detergents are needed for solubilization. In any case, it is essential that a suitable solvent is selected for protein extraction.

In all purification steps it is recommended that the pH of the protein solution is controlled, to avoid denaturation or deactivation of the target biomolecule. It is recommended that an operational pH condition in which the target protein presents maximum stability is determined, although this value may be different from the optimal for protein extraction.

Several factors affect the selection of the buffer solution, such as: the optimum pH; the buffer anionic or cationic species (which can interfere in the subsequent purification steps); the pH variation with ionic strength or temperature; the buffer reactivity with the proteins in solution; the biological activity (e.g. phosphates can inhibit or activate a protein in biological reactions); the interaction of the buffer with other components; the buffer permeation in biological membranes; the toxicity; the light absorption at 280 nm; the cost (especially if used in large-scale processes); and the protein solubility.

In many cases the target protein is bound to membranes or particles or is aggregated as a consequence of its hydrophobic characteristics. In these cases, detergents and chaotropic agents can be used to weaken these interactions during cell disruption and extraction steps. The detergent performance is highly dependent on pH and temperature.

Intracellular proteins usually expose thiol groups, which can oxidize during the purification process. Such groups may be protected by reducing agents such as 1,4-dithiothreitol (DTT), 1,4-dithioerythritol (DTE), and mercaptoethanol. Usually, reagent concentrations of 10–25 nM are sufficient to protect the thiol groups without reducing the internal disulfide bonds.

The presence of metal ions may be harmful to a biologically active protein owing to two main factors; the increase in oxidation of the thiol groups by molecular oxygen, and the complexation with specific groups. The chelating agent most commonly used to complex these metal ions is EDTA (ethylenediaminetetracetic acid) at a concentration of 10–25 nM. However, in some cases, application of metal ions such as calcium and magnesium may be needed to stabilize certain proteins.

The presence of proteases is a threat for protein stability. A simple strategy for protection against proteolytic degradation is based on the fast handling of the sample and use of low temperatures. One additional precaution is the addition of protease inhibitors, especially during disruption and extraction steps. In some cases, there is a need for a combination of inhibitors. Sometimes, pH adjustment is effective in inactivating proteases without affecting the stability of the protein product.

Despite being more critical for intracellular products, protease action may also occur in extracellular media. For example, in the expression of a baculovirus vector, which is a lytic system, proteases are often expressed and secreted during recombinant protein production, and the problem may assume a critical level. A similar problem occurs in serum-free culture systems. The absence of proteins such as albumin and macroglobulin reduces the protection against proteolysis. In these cases, addition of an antiproteolytic agent to the culture medium is recommended.

In order to avoid microbial growth in the protein solution during the purification process, buffer solutions should be routinely sterilized by filtration. Buffers such as phosphate, acetate, and carbonate at neutral pH are highly susceptible to microbial growth. Buffers with pH values lower than 3 or higher than 9 usually prevent bacterial growth, but can allow fungal growth. To circumvent such problems, it is recommended to proceed with the purification steps as fast as possible, always using fresh and filtered buffers, storing solutions at 4°C and, if possible, adding an antimicrobial agent to the buffer solutions.

12.4 Protein purification methods

After the extraction of the target biomolecule, purification is the next step. It is possible to select the entire sequence of purification steps for isolating a particular protein based on its properties, and on the fact that every protein presents a unique combination of characteristics. A strategy should be established to minimize the number of stages, leading to the maximum protein yield, minimum cost, and the required purity for the product's final application.

Table 12.1 shows the most commonly used purification techniques and related protein properties to provide separation. Every technique should be evaluated with regard to different parameters such as:

(i) the capacity, i.e. the sample amount that can be handled;
(ii) the resolution, i.e. how efficiently proteins are separated from each other;
(iii) the yield;
(iv) the cost.

A balance should be made between process parameters and performance expectation of each purification stage. In the early stages, capacity and cost are important parameters, whereas in the final stages, high resolution is more relevant.

The sequence of stages in a purification process usually starts with low resolution steps such as precipitation and liquid–liquid extraction, which

Table 12.1 Characteristics of typical purification methods: properties on which separation is based and their performance parameters

Principle of separation	Technique	Capacity	Yield	Resolution	Cost
Solubility	Liquid liquid extraction	High	High	Low	Low
	Fractional precipitation	High	High	Low	Low
Size	Microfiltration, ultrafiltration, dialysis	High	Medium	Low	Low
	Molecular exclusion chromatography	Medium low	High	Medium low	Medium
Electrical charge	SDS PAGE	Very low	High	High	Medium
	Electrofocusing	Very low	High	High	Medium
	Ion exchange chromatography	Medium	Medium	Medium	Medium
Specific interaction with ligands	Affinity chromatography	Medium low	Low	Very high	High
Surface hydro phobicity	Reverse phase chromatography	Medium	Medium	High	High
	Hydrophobic interaction chromatography	Medium	Medium	Medium	Medium

Adapted from Wheelwright, 1991.
SDS PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis.

are low cost and allow the processing of large amounts of material and high total protein concentrations. In the intermediate steps, a combination of chromatographic techniques is usually employed, followed by a final purification step focusing on the removal of product-related impurities (e.g. aggregates or isoforms) and/or those existing at very low concentrations. In this final stage, affinity chromatography and molecular exclusion are very usual processes.

The currently most relevant separation and purification techniques for processing biomolecules from animal cell cultures are discussed in the sections that follow. They are classified according to the protein characteristic on which the separation is based, such as solubility, molar mass, electrical charge, adsorption properties, and biological affinity for ligands.

12.4.1 Separation processes based on solubility

Protein precipitation

Precipitation is used as a separation step during the early stages of a purification process, usually followed by chromatographic separations, and also as a concentration method prior to an analysis or to a subsequent purification step.

The solubility of a protein in an aqueous solution relies on the distribution of the hydrophilic and hydrophobic groups on its surface. Protein precipitates are formed by the aggregation of protein molecules caused by changes in pH, ionic strength, or solvent dielectric properties, as well as by the addition of a miscible organic solvent, other inert solvents, and

polymers. Precipitation is based on the fact that proteins are electrolytes of high molar mass. The method is suitable for fractionation of protein mixtures, since each protein is composed of different amino acids that define its behavior. The precipitates can be recovered by filtration or centrifugation, and the remaining traces of both the precipitating agent and the redissolved precipitate can be removed by dialysis, diafiltration, or desalinization in a molecular exclusion column. Detailed reviews on the topic are given by Scopes (1994) and Kumar *et al*. (2003).

The most widely used precipitation agents for protein purification are ammonium sulfate and polyethylene glycol (PEG), which are discussed below.

Salting-in and salting-out

Neutral salts have a pronounced effect on the solubility of proteins, especially if they are globular. At low concentration, salts increase the solubility of many proteins in a phenomenon known as salting-in. This solubilization is a function of the solvent ionic strength, which depends on the concentration and on the electrical charge of the cations and anions that constitute the salt. These effects are caused by changes in the ionization of dissociating groups of the protein.

However, as the ionic strength is gradually increased, protein solubility begins to decrease. At high ionic strengths, the protein may be precipitated from the solution. This effect is known as salting-out. This phenomenon occurs when a salt is added to a system and the ions are solvated by water molecules displaced from the protein. Therefore, at high salt concentration the hydrophobic protein groups are exposed, enabling the formation of hydrophobic interactions between the protein molecules, thus causing aggregation. Larger proteins or those with more available hydrophobic regions will aggregate faster and this can be the basis of fractionation. The effectiveness of the salt is largely dependent on the anion, multivalent anions being the most effective. The order of selectivity may be described by the Hofmeister series (Ersson *et al*., 1998):

Anions: $PO_4^{3} > SO_4^{2} > CH_3COO > Cl > Br > NO_3 > ClO_3 > I > SCN$

Cations: $NH_4^{+} > K^{+} > Na^{+} > C(NH_2)_3^{+}$

Ammonium sulfate is frequently used for protein precipitation, due to its high solubility in water, which allows the solvent to reach very high ionic strengths. Usually, protein precipitates produced through salting-out do not denature and the protein activity is easily recovered. Addition of neutral salts can also protect proteins against proteolysis and microbial contamination.

Precipitation with polymers

Precipitation of proteins by the addition of anionic or cationic polymers occurs through the neutralization of charges and formation of high molar mass aggregates. Polymers commonly used for this purpose are carboxymethylcellulose and chitosan. Neutral polymers, such as PEG and methylcellulose can also be employed. In such cases, the mechanism is

similar to precipitation with organic solvents, that is, by change in the dielectric constant of the medium, although at lower concentrations (around 20%). Higher concentrations result in viscous solutions, making the recovery of the precipitate difficult. Polymers used in protein precipitation are expected to:

(i) present reversible solubility response to medium changes in pH and temperature;
(ii) form compact precipitates;
(iii) have low cost; and
(iv) be inert and nontoxic.

The polymer must have a high molar mass. PEG at a range of 6–20 kDa is commonly used (Ersson *et al*., 1998). Precipitation with PEG was used, for instance, by Deml *et al*. (1999) as one of the purification steps for the isolation of hepatitis B virus surface antigen (HBsAg) from the insect cell culture supernatant of genetically modified DS-2 (*Drosophila melanogaster* Schneider-2) cells.

Liquid–liquid extraction

Extraction is based on the transfer of a solute from one phase to another, according to its partition properties between two immiscible phases. In biotechnological processes, the most widely used extraction is liquid–liquid involving the partitioning of biomolecules, organelles or cells between the phases (Harrison *et al*., 2003).

In a bioprocess, two types of liquid–liquid systems are used. When molecules are stable in organic solvents, as occurs with low molar mass molecules (such as antibiotics), the extracting liquid can be an organic solvent immiscible with the aqueous phase originally containing the target biomolecule. However, in the case of proteins with a tendency to denature or degrade in the presence of organic solvents, aqueous two-phase systems are used (Franco *et al*., 2005). Such systems are usually formed by a polymer phase and a salt solution, or by two polymers, both soluble in water but immiscible with each other. The most commonly used systems are formed by PEG in one phase and a salt or other polymer (such as dextran) in the other. Under ideal conditions, the protein of interest is recovered in the upper phase (usually PEG) and the contaminants and particles (cells, fragments of cells, and organelles) in the lower phase (Ersson *et al*., 1998).

The partitioning coefficient K (equation 1) describes the distribution of a solute, in equilibrium, between two liquid phases. This coefficient is a function of several factors, such as the molecular size, pH, temperature, concentration, and type of components in both phases. In Equation (1), y and x are the solute concentrations in the extract and in the original solution, respectively.

$$K = \frac{y}{x} \tag{1}$$

Ideally, K should be maximized with a minimum volume of the extracting phase. Partition coefficients close to 1 implicate in large solvent

volumes and many consecutive extractions to achieve total recovery. A value of *K* close to zero, on the other hand, means that no extraction occurs (Harrison *et al*., 2003).

Liquid–liquid extraction offers several advantages, such as low cost, high yield, ease of continuous operation, and scale-up. Also, in the case of aqueous two-phase systems, the process would not cause degradation of proteins, enzymes, virus, or organelles (Cunha *et al.*, 2003; Harrison *et al.*, 2003). Efficient extraction techniques can lead to a reduction in volume, providing not only purification, but also a concentration of the sample. They are suitable for processing cell suspensions, enabling an integration of the solid–liquid separation and the primary purification stages and a consequent reduction in the number of stages in the downstream processing. However, resolution and purification factors are relatively low, compared with chromatographic techniques. Because of this, liquid–liquid extractions are often designed in the early stages of a purification process. Significant improvements in resolution can be achieved with the use of affinity ligands in one of the phases, although this can increase cost (Johansson, 1998).

12.4.2 Separation processes based on differences in molar mass

Proteins have high molar mass, the value of which differs from one protein to another. This allows the use of simple methods to separate proteins from low molar mass molecules, as well as to separate a specific protein from others.

Density gradient centrifugation

Just like other techniques, centrifugation can be applied at different stages in the downstream processing of a given protein. The main application of centrifugation in biotechnology (as seen in Chapter 11) is in the clarification of protein-containing suspensions, usually for the removal of cells and cell debris, but also after flotation and precipitation operations. However, density gradient centrifugation or zone centrifugation widens the range of possible applications of centrifugation by allowing the separation of small bioparticles such as organelles and viruses, as well as proteins. In the most widely used method, a continuous density gradient of sucrose is prepared with a device that adds a concentrated solution of sucrose to water, in decreasing proportions along a tube. In this way, the medium density is higher at the bottom of the tube, and the macromolecules to be fractionated are applied on the top. After centrifugation, the different proteins form bands or layers, according to their molar mass, shape, and density. Two consecutive centrifugation steps (the first using CsCl and the second using sucrose) were employed by Deml *et al*. (1999), for purifying HBsAg from cell culture supernatant of genetically modified DS-2 cells.

Dialysis

Desalting or buffer exchanges are often required between purification steps. At the laboratory scale, the protein solution is placed in a tube of a semipermeable polymer membrane immersed in the desired buffer. The membrane pore size determines the minimum molar mass of the compounds that are retained. Small molecules with a molar mass below the membrane cut-off will flow freely across the membrane until the osmotic pressure equilibrium is reached. Complete buffer exchange requires several changes of the dialysis liquid. The process should be carried out at a temperature around 4°C, to avoid loss of activity.

In industry, dialysis is not widely used because it is slow and labor-intensive. Here, desalting and/or buffer exchange is usually performed by diafiltration or molecular exclusion chromatography, which are discussed later.

Microfiltration

The principle of microfiltration is the application of hydrostatic pressure on a microporous filter membrane, so that the pressure difference forces solutes, water molecules, and particles smaller than the membrane pore size to flow across the pores, retaining and concentrating the larger particles in the suspension.

Microfiltration membranes usually have a nominal pore diameter in the range of 0.1–10 μm. However, the membrane specification is not an absolute parameter. The membranes usually present a pore size distribution around the nominal value and the shape of the bioparticles can determine whether they are retained or pass through the membrane. The membranes are manufactured from polymers, such as Teflon®, polyester, PVC (polyvinyl chloride), Nylon®, polypropylene, polyethersulfone, and cellulose, or from inorganic materials, such as ceramic and sinterized stainless steel.

In microfiltration, the permeate flux increases inversely with the suspension viscosity and proportionally to the applied pressure, provided that there is no membrane fouling (Belford, 1988; Ho and Zydney, 2000). To accelerate the process, it is possible to decrease the solution viscosity by increasing the temperature, although not so much as to denature the protein.

Microfiltration is widely used for the removal of cells and fragments from suspension. It is also used as a method of sterilization of solutions, and has the advantage of high efficiency, simplicity, compactness, and reliability.

Ultrafiltration

In ultrafiltration, water and other low molar mass molecules are forced through a semi-permeable membrane by the application of high pressures (1–7 bar) or of a centrifugal field. This technique involves membranes with pore diameters in the range of 1.0–20 nm, which are most commonly characterized and selected based on their nominal molar mass cut-off

(MWCO), usually defined as the molar mass at which the membrane rejects 90% of solute molecules. However, as in microfiltration, the molecular shape can affect permeability through the membrane pores. For example, a membrane with a nominal cut-off of 100 kDa, which does not allow globular molecules with a molar mass of 100 kDa to flow through, may allow fibrous molecules with higher molar masses to flow across the pores. As in microfiltration, the membrane pore size is not uniform, with a normal distribution around an average value.

A common problem of this technique is the gradual decrease in permeate flux associated with membrane clogging or fouling, caused by adsorption or physical deposition of particles and/or macromolecules on membrane pores. Fouling can be minimized by prior clarification (particulate removal) of the feed solution, by the selection of operational conditions that minimize interactions between membranes and macromolecules, by the use of tangential flow, or by performing intermittent back-flushing operations.

Diafiltration

Diafiltration consists of the application of microfiltration or, more commonly, ultrafiltration membranes for solvent exchange. In diafiltration systems, schematically shown in *Figure 12.2*, the permeate is collected continuously at the same rate at which fresh solvent is added, so that undesirable solutes are removed in the permeate stream and the retentate volume is kept constant. Although diafiltration is being increasingly used, especially on a large scale, a disadvantage is the generation of large volumes of liquid effluents. For instance, in a system with a 100 m^2 membrane operating at a permeate flux of 30 $L\,m^{-2}\,h^{-1}$, a volume of 9210 L of water is required to remove 99.99% of the salt present in a protein solution (Harrison *et al*., 2003).

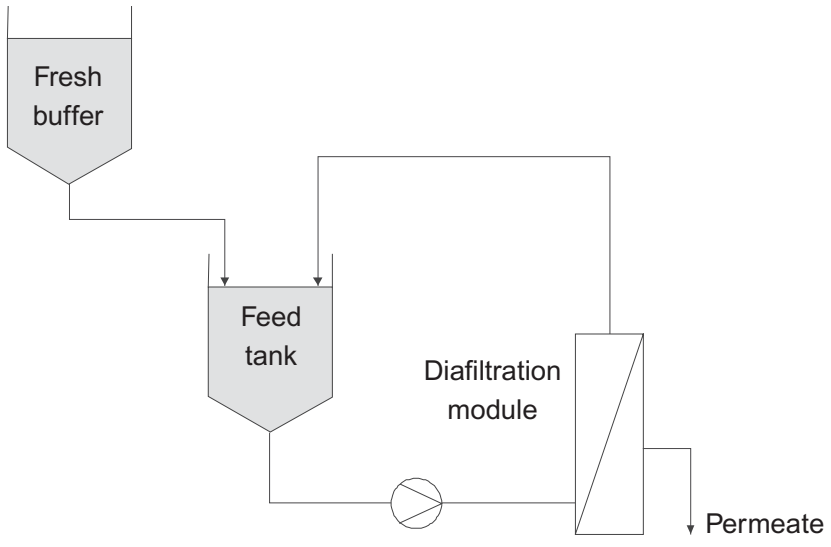


Figure 12.2

General scheme of a diafiltration system.

Molecular exclusion chromatography

Molecular exclusion chromatography, also known as gel filtration, size exclusion chromatography, gel permeation chromatography, or simply gel chromatography, is another separation method based on differences in molecular size.

In this method, molecules partition between a solvent (aqueous buffer) and a stationary phase of defined porosity. A protein mixture dissolved in a suitable buffer flows through a column packed with spherical porous particles made of an inert material (usually a polymer or a gel). The column is equilibrated with a pre-selected buffer appropriate for sample elution. The flow occurs by gravity or aided by a pump.

In a sample containing a mixture of molecules, the smallest permeate the matrix pores and follow a slow trajectory along the column axis and are collected later forming the later chromatogram peaks. The largest molecules are excluded from the pores, migrating through the interstitial space and are, therefore, eluted earlier. The intermediate sized molecules may partially penetrate the pores, allowing intermediate elution times.

Therefore, the molecules are eluted in the inverse order of their molecular size, as indicated in *Figures 12.3* and *12.4*, and the differences in the elution times of different proteins are related to the fraction of pores accessible to the solutes. Equations can be obtained that relate the fraction of pores of different dimensions, the gel structure, and the molecular size of solutes with so-called distribution coefficients.

The distribution coefficient K_d (Equation 2) is defined as the volume fraction of pores, in a stationary phase, which is effectively permeated by a solute of a given size. V_o is the interstitial volume of the porous medium, measured by the elution volume of a high molar mass solute that is totally excluded from the matrix pores. V_e is the elution volume of the product of interest. V_S represents the total solvent volume within the pores, available for small solutes.

$$K_d = \frac{(V_e - V_o)}{V_S} \qquad (2)$$

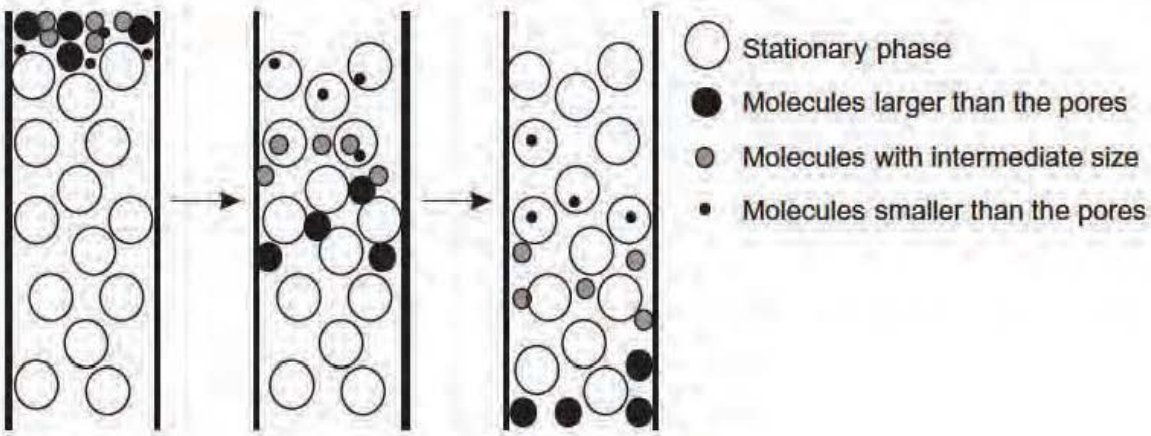


Figure 12.3

Schematic illustration of the separation principle involved in molecular exclusion chromatography.

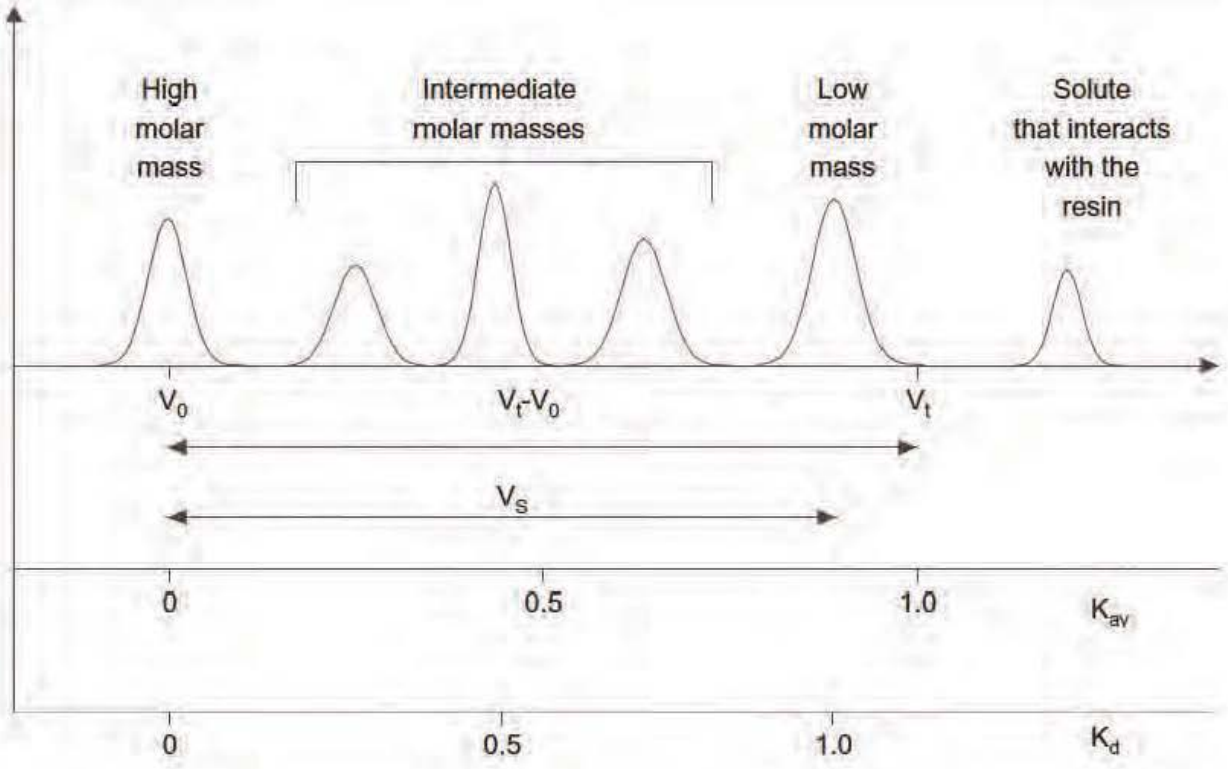


Figure 12.4

Elution profile of a mixture containing solutes of different molar masses and the variables used to describe solute and matrix behavior (adapted from Ladisch, 2001).

Since V_S is difficult to measure, it is common to use an alternative distribution coefficient, called K_{av} (Equation 3). In this approach, V_S is replaced by the difference between the total column volume (V_t) and the interstitial volume (V_o) of the column packed with the chromatographic matrix (Ladisch, 2001; Moraes and Rosa, 2005).

$$K_{av} = \frac{(V_e - V_o)}{(V_t - V_o)} \tag{3}$$

For a given matrix, K_d or K_{av} can be plotted against the log of the molar mass of different solutes. For a given molar mass range, a linear relationship will be observed. Therefore, by applying to the column molecules of known molar mass, and similar shape and density, it is possible to determine the molar masses of solutes present in a sample (Kumpalume and Ghose, 2003; Moraes and Rosa, 2005).

Ideally, the column matrix should be inert with regard to the molecules to be separated. If electrostatic or hydrophobic interactions occur between proteins and the stationary phase, it is recommended, respectively, to increase or decrease the ionic strength of the liquid phase, otherwise partial adsorption of the protein may cause a delay in elution from the column. This would cause a longer elution time than that expected based on the molar mass.

The most commonly used matrices for molecular exclusion chromatography are cross-linked polymer gels, such as agarose, dextran, and polyacrylamide. Since dextran and agarose are biodegradable, they should be stored in the presence of antimicrobial agents.

The selection of a gel matrix should take into account pH and temperature stability, which should be compatible with the characteristics of the target protein. The selection of the most suitable gel should also take into account the main goal to be achieved. If the process is intended to separate proteins from low molar mass solutes (< 5 kDa), a small pore size matrix is recommended, so that proteins are completely excluded from the porous medium. Such a strategy is used for desalting samples.

Matrices composed of small particles have high resolution, since the molecular diffusion in the interstitial region is small and, therefore, peak broadening is low. However, these systems are associated with low flow rates, since small particles result in larger pressure drops along the column. Because the application of high pressure can lead to particle deformation and, consequently, to bed compaction, particles that are large and rigid, capable of withstanding high flow rates, are commonly preferred for large-scale processes, even if they are associated with lower resolution.

In molecular exclusion chromatography, resolution is directly related to the sample loading volume, since the higher the sample volume, the higher the volume in which the protein is eluted. Usually, sample volume should be 0.5–5% of the total bed volume. For volumes below 0.5% the sample becomes too diluted, while volumes above 5% work well only for molecules with large differences in size. For desalting, due to the pronounced difference in the molecular size of the components, sample volumes can reach up to 30% of the column capacity without a significant decrease in resolution.

With regard to the total protein concentration in the injected sample, it should be ensured that the ratio between the viscosity of the sample and the eluent is not higher than 2. High viscosities can cause problems, such as the occurrence of regions with distorted and irregular flow patterns. Hence, protein concentration in the loaded sample should ideally be in the range of 10–20 mg mL ^{1}, although in some cases it can reach 70 mg mL ^{1}.

Therefore, to achieve high resolutions in molecular exclusion chromatography, it is common to use columns with large height-to-diameter ratios (in the range of 20–40), although at an industrial scale this can become difficult.

12.4.3 Separation processes based on differences in electrical charge

Separation of proteins based on differences in their electrical charge depends on their acid-base properties, which are mostly determined by the number and type of ionizable side groups in the peptide chain. Since proteins are different from each other with respect to their composition and amino acid sequence, they also have distinct acid-base properties. Information on these properties allows a prediction of the behavior of a given protein when exposed to an electrical field.

The acid-base properties of proteins are exploited in two methods, electrophoresis and ion exchange chromatography. These are widely used in the analysis and separation of protein mixtures.

Electrophoresis

Protein molecules are electrically charged at pH values different from their isoelectric point (pI). As a consequence, they can migrate when exposed to an electrical field, at a rate dependent upon their electrical charge densities.

In the separation of proteins by electrophoresis, the sample is submitted to an electrical field, causing the electrically charged proteins to move in the direction of the applied current. If the experiment is carried out in solution (free electrophoresis) and the proteins have different charge densities, they will move with different velocities, allowing their separation. In practice, the process is normally carried out in a gel matrix, instead of in solution, and the gel can act not only as an inert support for the electrophoresis buffer, but also, if desired, as an active material that interacts with the proteins.

A very popular electrophoretic technique is SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis), used for separating protein molecules based on their molar masses, providing high resolution and also allowing the determination of the protein molar masses. In this method, the sample is pre-treated with the detergent SDS, which causes the dissociation of protein subunits and the complete unfolding of the polypeptide chain, which interacts with SDS molecules forming elongated rod-shaped complexes. This provides identical charge/size ratios to all proteins, so that the migration velocity of the denatured proteins becomes dependent only on the molar mass.

Another version of electrophoresis, that is also very efficient for protein separation, is isoelectric focusing or electrofocusing. In this technique, a protein mixture is submitted to an electrical field in a gel support, such as polyacrylamide or agarose, with a gradient of increasing pH established from anode to cathode. Since the loaded proteins will be positively charged at pH values below their pI, and negatively charged at pH values above their pI, a protein will migrate until it finds a location within the gel where the pH is the same as its pI (Låås, 1998).

Electrophoretic techniques have a number of practical advantages, such as high resolution, equipment of relative simplicity and low cost, feasibility for multiple samples, high sensitivity, easy detection of specific molecules, and availability of standards giving reasonably well defined bands. However, as a consequence of its low capacity, electrophoresis is mostly used for analytical purposes rather than as a preparative technique. More details on electrophoretic techniques are provided in Chapter 13.

Ion exchange chromatography

Among the chromatographic methods, ion exchange is the most commonly used in protein purification, due to its simplicity for scale-up, wide applicability, and low cost compared with other chromatographic methods. Ion exchange of proteins involves their adsorption onto the charged

groups of a solid support, followed by their elution and concentration in an aqueous buffer of high ionic strength.

For the effective use of ion exchange for protein purification, the stationary phase should be able to bind to positively or negatively charged proteins. There are two types of ion exchangers: anion exchangers (positively charged matrix) and cation exchangers (negatively charged matrix). Low molar mass counter-ions are associated with the proteins, as well as with the stationary phase. For protein binding onto the stationary phase, the counter-ions should be dissociated. The most widely used counter-ions are Na^+ and H^+ in cation exchangers, and Cl and OH in anion exchangers.

The counter-ions can be arranged according to their intensity of interaction with the other ionic groups at the same concentration. Consequently, a chloride would displace hydroxide ions as a counter-ion in ion exchange. The counter-ions are not permanently bound to an ionic group, but are in an equilibrium state, where continuous replacements take place. So, ionic groups can become free to bind a protein. The higher the counter-ion concentration, the lower will be the probability of the ionic groups being available for protein binding. Sometimes, before the ion exchange process, counter-ions should be replaced by others more suitable for the specific application.

The support can be cellulose, agarose, dextran, silica, polyacrylate, polyvinyl, or polystyrene, among other resins. The most suitable matrix for a given application is often selected based on data provided by the manufacturers.

The selection of the ionic functional groups of the support is based on the strength of these groups, as listed in *Table 12.2*. The most widely used functional groups are S, C, DEAE, and Q. The functional groups S and Q are a strong acid and a strong base, respectively. Their pK values are around 1 and 14, respectively, and thus, they are completely ionized at practically any pH. The functional groups C and DEAE are a weak acid

Table 12.2 Chemical groups used in ion exchange for protein purification

Formula	Group
Strong anion exchangers	
$CH_2N^+(CH_3)_3$	Trimethylaminomethyl (TAM)
$C_2H_4N^+(C_2H_5)_3$	Triethylaminoethyl (TEAE)
$CH_2N^+(CH_3)_3$	Quaternary amine (Q)
Weak anion exchangers	
$C_2H_4N^+H_3$	Aminoethyl (AE)
$C_2H_4N^+H(C_2H_5)_2$	Diethylaminoethyl (DEAE)
Strong cation exchangers	
SO_3^-	Sulfonate (S)
$CH_2SO_3^-$	Sulfomethyl (SM)
$C_3H_6SO_3^-$	Sulfopropyl (SP)
Weak cation exchangers	
COO^-	Carboxy (C)
CH_2COO^-	Carboxymethyl (CM)

From Ladisch (2001), Karlsson *et al.* (1998).

and a weak base, respectively, with pK values around 4 and 10. For the weak groups, the prevailing pH affects the ionization, and the pK is used as an indication of the adequate operational pH range. Therefore, to ensure a suitable ionization, the cation exchanger C is used at a pH above 6, and the anion exchanger DEAE at a pH below 9.

Besides influencing the adsorbent ionizing groups, the pH also affects protein charge and stability. In practice, if the protein is more stable at a pH below its pI, a cation exchanger is used, and conversely, if it is more stable at a pH above its pI, an anion exchanger is used.

Some adsorbents present a remarkably high density of ionic groups on the surface, allowing multiple adsorption points, which require high salt concentrations to promote elution, which may cause denaturation.

The aqueous buffers used in ion exchange should contribute to the ion/counter-ion dissociation. The buffer minimizes pH fluctuations, avoiding protein denaturation. The selection of the most suitable buffer depends on the type of the ion exchanger, on product stability, and also on the optimum pH for maximum adsorption. An important requirement is that the buffer should not interact with the adsorbent and this is the reason why the selected buffer, when charged, usually has the same charge as the ion exchanger. Depending on the chromatographic step, adsorption or elution, the pH should be adjusted to promote protein adsorption or its displacement from the matrix.

During adsorption, the pH should be adjusted to one unit above or below the protein pI, since larger differences result in a greater net charge of the protein, and consequently, multiple adsorption points, requiring severe conditions for elution. An ideal pH is suitable for adsorption at a level which allows the elution to be performed only by a small pH change.

The buffer ionic strength determines the degree to which the ionic groups of both the protein and stationary phase are blocked. During adsorption, the highest ionic strength that still enables adsorption of the desired protein is used, whereas during elution, the lowest ionic strength that promotes its desorption is recommended. If the ionic strength is excessively low during adsorption, the protein will adsorb very strongly, making elution difficult. Keeping the ionic strength as high as possible during adsorption minimizes the adsorption of contaminants, and keeping it low during the elution minimizes the desorption of contaminants. Such a strategy simplifies the elution step.

Once the optimum adsorption/desorption conditions are established, other issues should be considered, such as the need for matrix pretreatment and the operational mode for adsorption and elution steps. Pretreatment of the ion exchanger can involve, for example, the removal of fine particles, swelling, washing, or counter-ion replacement.

Adsorption can be carried out in tanks and in a batch system, as well as in chromatographic columns. Batch adsorption can be carried out in the early purification step, allowing the processing of large product volumes, despite having a low efficiency. Column adsorption, on the other hand, presents limitations with regard to the flow rates, but gives better resolution. There are two distinct methods of elution. The first method is that protein adsorbed on the static ion exchange matrix is completely eluted by a small volume of a strong eluent. This method is useful for the concentra-

tion of a protein present in a large sample volume. The second method involves a dynamic ion exchange in which protein separation relies on relative migration velocities along the column. In this case, there are three distinct modes of elution:

(i) isocratic, employed for weakly bound proteins, resulting in large elution volumes;
(ii) in steps, based on discontinuous and sequential changes of pH and/or salt concentration;
(iii) gradient, based on continuous changes in the eluent composition (pH and/or ionic strength).

The protein concentration in the eluted fractions collected from a chromatography column is frequently determined by measuring the absorbance in the UV region (usually at 280 nm).

An advantage in ion exchange chromatography is that the matrix can be regenerated by the removal of bound contaminants and by the reconstitution of the counter-ions, providing the matrix in the condition required for a new protein adsorption cycle. If the support matrix is stored wet, it is susceptible to microbiological degradation, and the use of antimicrobial agents during storage is recommended.

12.4.4 Separation processes based on differences in hydrophobicity

The surfaces of proteins are mostly hydrophilic. Although the majority of the hydrophobic residues tend to be buried in the interior of the protein, some hydrophobic regions are also found on the surface (Voet and Voet, 1995; Ladisch, 2001). The level of surface hydrophobicity differs from one protein to another, mainly as a consequence of the amino acid composition and sequence. Difference in surface hydrophobicity is the property exploited in hydrophobic interaction chromatography (HIC) and reverse phase chromatography (RPC).

In both chromatographic methods, the adsorbent contains hydrophobic groups. However, in RPC adsorbent hydrophobicity is much higher than that in HIC (Eriksson, 1998). Therefore, in HIC the adsorbent is a weak hydrophobic phase, whereas in RPC it is a dense hydrophobic phase.

Hydrophobic interaction chromatography

The adsorbents used in hydrophobic interaction chromatography are usually polymer resins, such as reticulated agarose, derivatized with aliphatic groups such as butyl and octyl, or with aromatic groups, such as phenyl.

The tendency for protein binding to hydrophobic groups is often reduced in low salt concentrations. Therefore, to increase the hydrophobic interaction intensity, high salt concentrations are used during equilibration and sample application. Due to their effect on strengthening the hydrophobic interactions, the most effective salts are those used in salting-out precipitation, such as ammonium sulfate.

For the selection of the operational conditions, it is recognized that hydrophobic interactions can be weakened by several factors, such as a temperature decrease, pH change, presence of organic solvents, non-ionic detergents, and polyols such as PEG.

Usually, desorption is performed by means of a decreasing salt gradient, so that the solutes are eluted according to the increasing order of their surface hydrophobicity. The remaining proteins can also be eluted by the application of buffers with increasing concentrations of, for example, PEG. The operational conditions during the process are mild, since the salts used in HIC exert a stabilizing effect on proteins (Ladisch, 2001).

HIC has a high adsorption capacity and provides high recoveries, making it a popular technique for large-scale applications (Kumpalume and Ghose, 2003). A limitation, however, is the high cost of using large amounts of salt. Its selectivity is not high, being lower than that of affinity chromatography. HIC is suitable when combined with ion exchange and molecular exclusion chromatography steps (Maugeri Filho and Mendieta-Taboada, 2005).

Reverse phase chromatography (RPC)

Currently, the most widely used adsorbents in RPC are silica resins, containing a hydrophobic phase, usually octyl (C8), octyldecyl (C18), methyl (C1), or phenyl groups. Additionally, new adsorbents based on organic materials such as methacrylate, polystyrene, and copolymers of styrene and divinylbenzene have been developed (Hearn, 1998).

Due to its high hydrophobicity, the adsorbent interacts strongly with proteins, requiring low salt concentration during the adsorption, and increasing gradients of organic solvents, such as methanol, isopropanol, and acetonitrile, during elution (Harrison *et al*., 2003).

Despite being widely used in protein analysis, peptide mapping, and for purification of low molar mass molecules, RPC is not often used for protein purification on a large scale (Ladisch, 2001).

12.4.5 Separation processes based on specificity of ligands

Some proteins function through specific non-covalent binding to other molecules, termed ligands. Ligands can be small molecules, such as enzyme substrates, or larger molecules, such as hormones. Protein interaction with the ligand is determined by the size and shape of the ligand, as well as by the number and distribution of complementary regions. These regions combine charged and hydrophobic portions, presenting other short range interactions, such as hydrogen bonds.

Protein–ligand interaction, which is stereo-specific and consequently presents high affinity, can be used for the isolation of a given protein from a complex mixture. This provides a high degree of purification. The most widespread technique is affinity chromatography. Affinity ligands have also been used to increase the resolution and the selectivity of other techniques such as precipitation (Lali *et al.*, 1998), liquid–liquid extraction (Johansson, 1998), and filtration assisted by macroligands (Romero and Zydney, 2002).

Affinity chromatography

Affinity chromatography is an adsorption method based on the recognition between a ligand immobilized on a solid matrix and a biomolecule to be separated. The main difference between this method and other chromatographic techniques is the high interaction specificity between molecules and stationary phase.

As shown in *Figure 12.5*, during the adsorption step interactions occur between the target biomolecule and the ligand located in the adsorbent

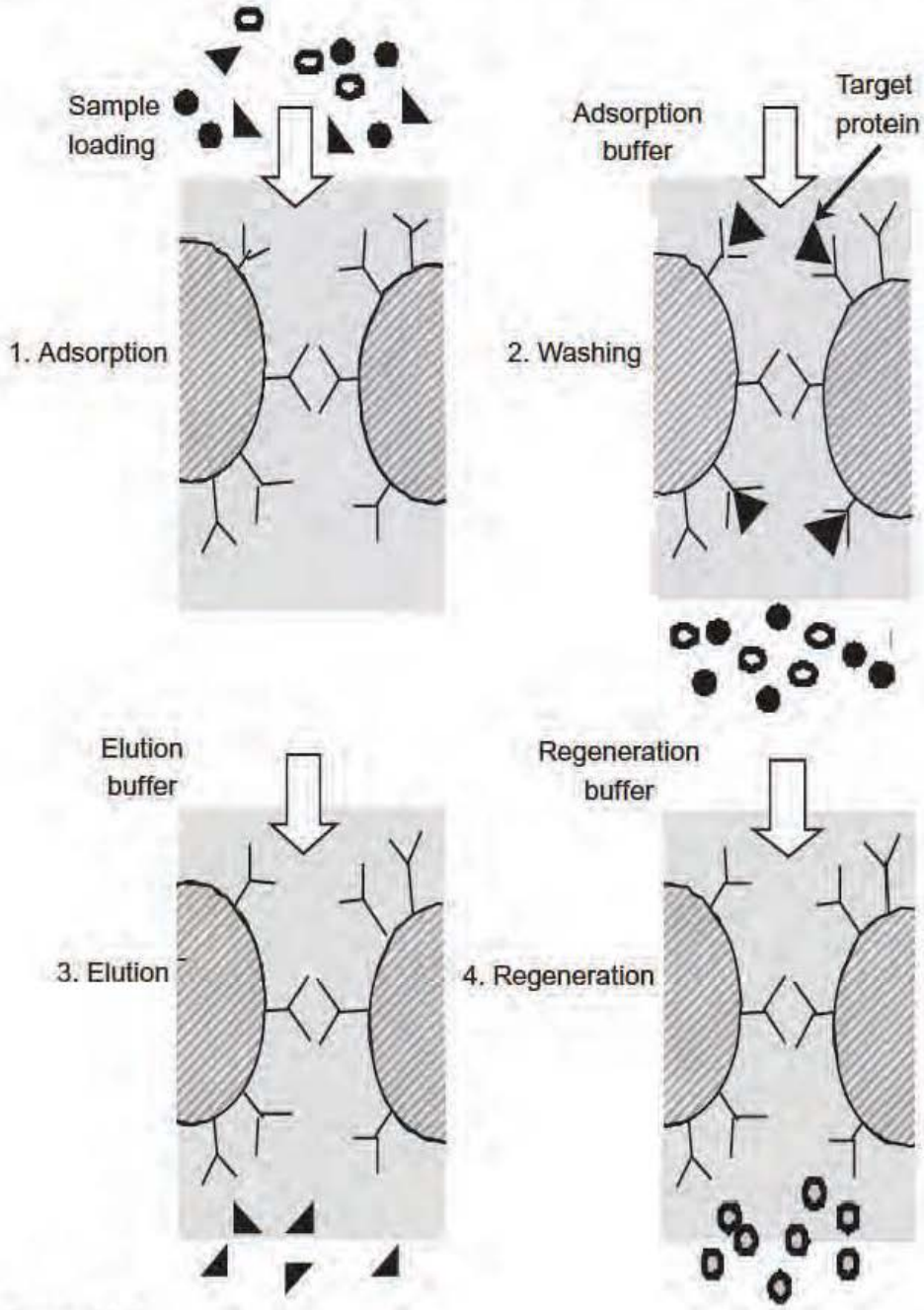


Figure 12.5

Principles underlying affinity chromatography.

pores, whereas the remaining molecules flow through the adsorbent without interacting. Next, in the washing step, a buffer (usually the same used in the equilibration or adsorption steps) is pumped through the column, for the removal of molecules retained in the interstitial space or weakly adsorbed by non-specific interactions. During the elution step, desorption of the target biomolecule is promoted by changes in the medium (pH, ionic strength, etc.) intended to weaken the interactions between the ligand and the target biomolecule, or by the addition of counter-ligands with a strong affinity to the ligand or to the adsorbate. Finally, regeneration is carried out by washing the column with a specific solution, to recover the adsorbent for its reuse in a new cycle.

Vijayalakshmi (1989, 2002) classifies the ligands used in affinity chromatography in two groups: biospecific and pseudo-biospecific. Biospecific ligands interact with the target biomolecule through functional affinity, exactly as happens naturally, for example in antigen–antibody, glycoprotein–lectin, and hormone–receptor interactions. Biospecific ligands provide high specificity and high adsorption capacities, with possibilities of achieving purification factors up to 1000-fold (Roper and Lightfoot, 1995). A common example of the application of biospecific ligands is the purification of monoclonal antibodies (mAbs) from cell culture supernatants using protein A and G as the ligands (Castilho *et al*., 2002a; Rasmussen *et al*., 2005; Hahn *et al*., 2006). However, biospecific ligands are usually high cost molecules with a fragile three-dimensional structure. The interaction can sometimes be so strong that adsorbate desorption is only possible under drastic pH or ionic strength conditions.

Pseudo-biospecific ligands, such as metal chelates, amino acids, and dyes are simpler and less expensive molecules with a structural affinity to biomolecules (group specificity). Since they have simpler structures, they can be immobilized by stable, well defined chemical reactions to the chromatographic matrix. Their disadvantages are their lower specificity, as compared with biospecific ligands. However, there are many examples of molecules obtained from animal cell cultures that have been successfully purified using pseudo-biospecific ligands (El-Kak and Vijayalakshmi, 1991; Atkins *et al.*, 2005; Serpa *et al.*, 2005; Kumar *et al.*, 2006).

Due to the central role of the ligand in affinity chromatography, some factors should be considered when selecting a ligand for the purification of a protein:

(i) specificity: the ligand should be able to selectively recognize the target protein;
(ii) reversibility: the ligand should form a reversible complex with the protein to be purified, and the complex should be resistant to the composition of the feeding stream or washing buffers, but easily dissociated during elution without denaturation;
(iii) stability: the ligand should be stable under the conditions employed during immobilization and chromatography;
(iv) immobilization feasibility: the ligand should contain a functional group suitable for covalent binding to the support, without affecting interaction properties with the protein.

The interaction between the ligand (L) and the protein (P) to form the reversible complex (PL) can be described by:

$$P + L \rightleftarrows PL$$

where the dissociation constant, K_D, is given by Equation (4):

$$K_D = \frac{[P][L]}{[PL]} \tag{4}$$

In general, in affinity techniques K_D values are in the range of 10^{-8} to 10^{-4} M, but the K_D determined for free ligands can differ from those determined for immobilized ligands. Many authors report their data in terms of the association constant (K_A), which is equal to K_D^{-1}.

After selecting the ligand, a suitable matrix should be chosen for its immobilization. The effectiveness of an immobilized ligand can be dependent on the matrix structure. Different criteria are used to select the solid matrix. The matrix should have high porosity and a suitable pore size, so that the target protein can access the ligands immobilized in its interior. It should be chemically stable during activation, ligand coupling, adsorption, elution, and regeneration. The matrix should also be mechanically resistant and rigid to allow suitable mobile phase flow, and should tolerate wide pH and temperature ranges. The matrix should have a uniform structure, possess high concentration of functional groups for ligand coupling, and should not promote non-specific adsorption.

For a particular application, the relevance of these criteria may change. Widely used matrices are agarose and synthetic polymers also used in ion exchange and molecular exclusion chromatography. The ligand can be coupled to the solid matrix through spacer arms, especially when the ligand is small and stereo hindrance can affect its interaction with the target protein. The length of the spacer molecule is important and, also, it should have reactive ends enabling its coupling to the matrix and to the ligand. The spacer molecule should not interact with proteins and should preferably be hydrophilic. The coupling of a ligand to a matrix by means of a spacer arm is based on two general procedures, described in *Figure 12.6*.

Once a suitable matrix is obtained, with the ligand coupled to it, and properly characterized with regard to ligand concentration, the effectiveness of the protein–ligand interaction can be evaluated. A sample containing the target protein is applied to the column, which is washed and evaluated for retained protein.

In affinity chromatography, there are several elution methods. One involves the elution by affinity based on the addition of high concentrations of a free ligand in the buffer. The eluted complex can later be dissociated by changing the buffer salt concentration or by the addition of a surfactant that decreases hydrophobic and van der Waals interactions, such as a non-ionic detergent. Another possibility is elution based on high salt concentration. In this case, non-hydrophobic interactions between the protein and the immobilized ligand are broken, including biospecific ionic forces and other polar forces. Other elution methods are based on pH and

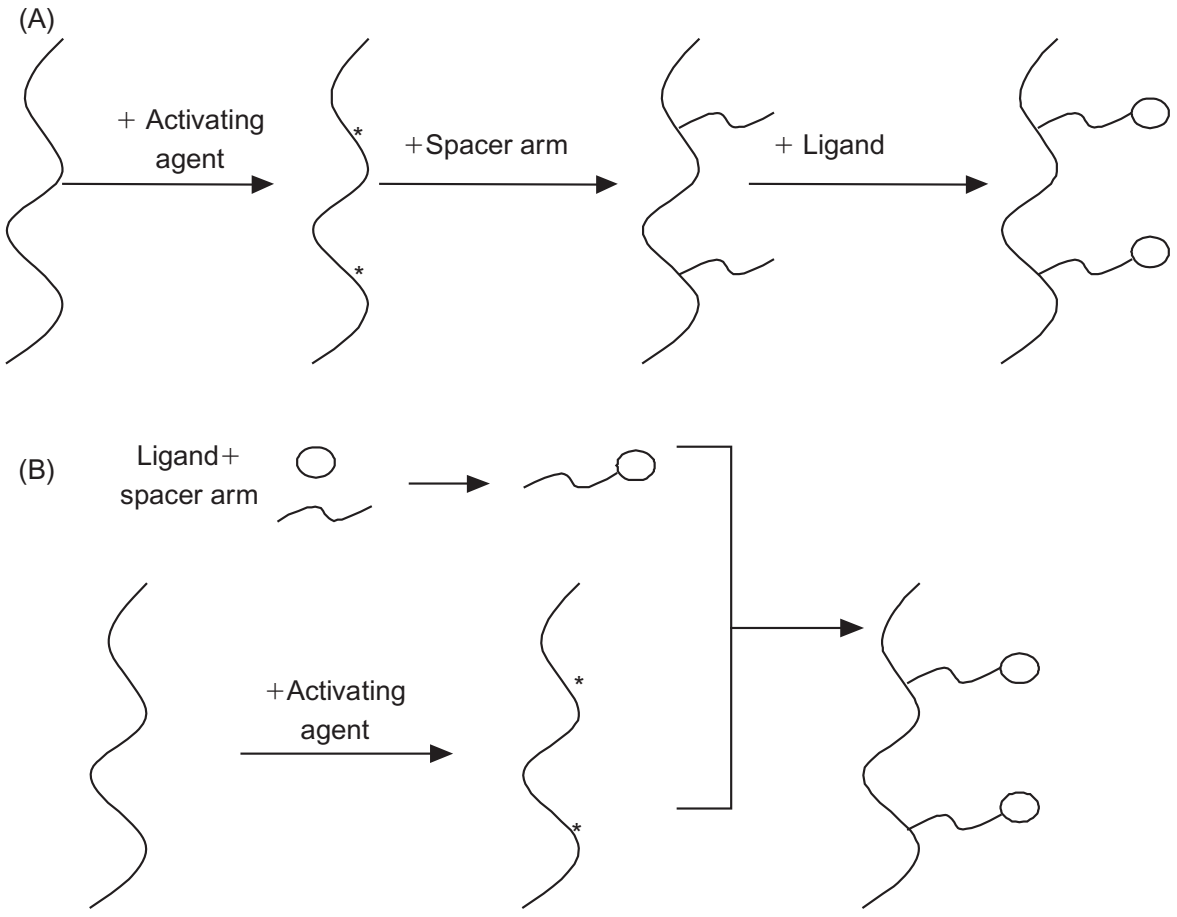


Figure 12.6

Alternative routes for the coupling of ligands to a matrix. (A) The spacer arm is coupled to the matrix and then the ligand is covalently attached to the support; (B) the spacer arm is first attached to the ligand, and then it is attached to the matrix.

temperature changes (lower temperatures weaken hydrophobic interactions) or on the addition of chaotropic agents.

A major concern in affinity chromatography is the release of the ligand from the matrix and its consequent loss in the column outlet stream. This can happen due to instability of the ligand–matrix binding or to dissolution of the matrix. Released ligands can contaminate the product and decrease the adsorbent performance. This reduces the possibilities for adsorbent reutilization, which has an impact on process costs.

For washing and storing, the majority of adsorbents used in affinity chromatography can be regenerated with solutions of high salt concentration (2 M KCl) and stored in the presence of antimicrobial agents.

Affinity chromatography offers several advantages over other types of chromatography because of its remarkable levels of purification and high yields, which can come close to 100%, at least at laboratory scale (Walsh and Headon, 1994). Because of its high selectivity, it allows the isolation of a molecule present in low concentration in a complex mixture, enabling the processing of large solution volumes and desorption with reduced

eluent volumes. This provides high purification and concentration factors in a single step, in short times, and with high recoveries of biologically active product.

12.4.6 Other developments

Expanded bed adsorption

Expanded bed adsorption (EBA) was derived from studies in the 1970s, aimed at carrying out chromatography techniques in fluidized beds (Kilikian and Santos, 2005). However, unlike fluidized beds, in an EBA column the bed expansion is stable and predictable. This occurs because the adsorbent usually consists of hybrid particles of high density such as quartz, covered with a polymeric material such as cross-linked agarose to which the immobilized ligands are attached (*Figure 12.7*; see color section). By manipulating the proportion of these two materials, an apparent density distribution in the range of 1.15–1.20 g cm ^{3} is obtained. The particles have a size distribution, usually in the range of 100–300 μm. When subjected to an ascending stream of the mobile phase, the particles segregate within the column at different equilibrium positions, according to their sizes and apparent densities.

In EBA, it is common to characterize the column and adsorbent through the experimental determination of expansion curves, which show bed expansion as a function of the superficial velocity of the ascending mobile phase. The degree of expansion is defined as the ratio between the height (H) of the expanded bed at a given superficial velocity, and the height of the packed bed (H_0), before pumping the mobile phase. The expansion curve of the adsorbent Streamline-rPrA in a Streamline Direct 24 column is shown in *Figure 12.8A*. For the characterization of the adsorption performance of a column, breakthrough curves can be determined for different superficial velocities (GE Healthcare/Amersham Biosciences, 2002), and for different size columns, if scale-up is intended.

After adsorption, the column is washed with the bed still in the expanded form, whereas elution is usually performed with the bed in the packed form (*Figure 12.8B*), to minimize the elution volume, and thus achieving a high concentration factor for the target protein.

EBA is basically an operation mode that is suitable for different types of chromatography, such as ion exchange, affinity, and hydrophobic interaction. Its main advantage is that, due to bed expansion, it allows the application of cell suspensions, without loss in chromatography performance (Anspach *et al*., 1999). Therefore, EBA allows the elimination of the solid–liquid separation steps that usually precede a chromatographic process, reducing the number of downstream steps, with a decrease in processing time and an increase in overall yield. Such advantages, combined with purification and concentration factors that are adequate for primary purification steps, explain why this technique has been used increasingly in recent years on laboratory as well as on an industrial scale.

Interesting results obtained on a pilot plant scale, for the direct purification of a mAb from 100 L of a hybridoma-containing suspension, were

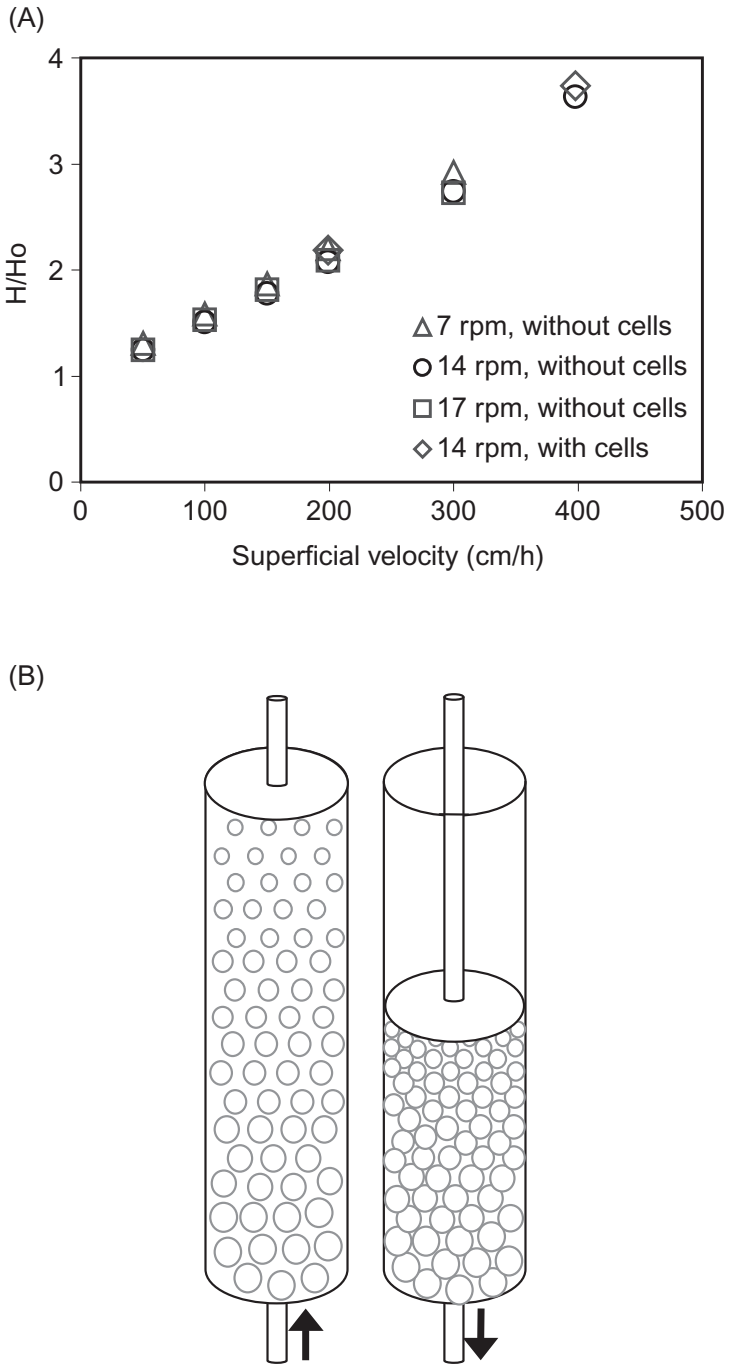


Figure 12.8

(A) Expansion curve of the adsorbent Streamline rPrA®, in a Streamline Direct 24® column, for different stirring velocities (located on the bottom of the column), in the presence or absence of cells in the feed. H: height of the expanded bed; H_o: height of the packed bed. (B) Illustration of the adsorption step (left) in expanded bed mode, and elution (right) in packed bed mode.

reported by Ameskamp *et al*. (1999). Examples of EBA applications in the industrial processing of vaccines and recombinant proteins from CHO cells have also been reported in the literature (GE Healthcare/Amersham Biosciences, 2000). For the direct application of cell suspensions, it is

recommended to use a specific column equipped with a stirrer positioned at the bottom, which helps to prevent aggregation of adsorbent particles and cells.

Membrane adsorbers

Membrane adsorbers derive from the technological developments in the membrane separation field and in column chromatography of proteins. They combine the high selectivity of chromatographic separations and the high productivity usually obtained in membrane separation processes.

It differs from conventional chromatographic adsorbents in that the support for ligand immobilization is composed of microporous membranes, usually polymeric, with a nominal pore size generally in the range of 0.4–3 μm. Depending on the nature of the immobilized ligand, the membrane is characterized as an ion exchange, affinity, or hydrophobic interaction adsorber (Thömmes and Kula, 1995; Charcosset, 1999; Haupt and Bueno, 2000; Klein, 2000; Bueno and Miranda, 2005). Like chromatography with resin matrices, the selectivity of separation is determined by the ligand–adsorbate pair and depends on pH, ionic strength, and temperature. Adsorption isotherms in *Figure 12.9* show the differences presented by a poly(sulfone) membrane containing the synthetic peptide TG19318 as the ligand, concerning the affinity for immunoglobulins (Ig) from different classes, in the presence of different buffers.

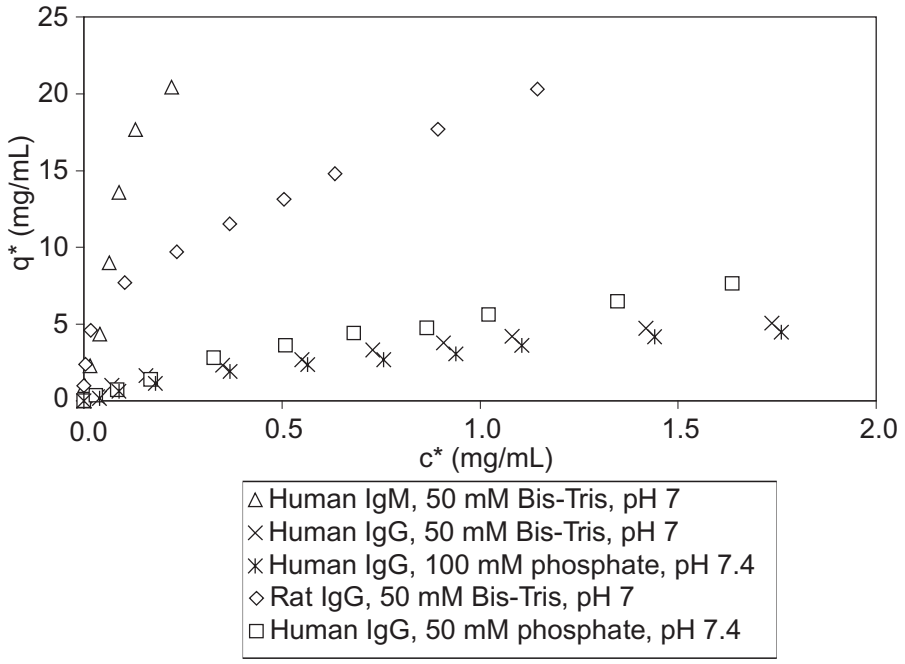


Figure 12.9

Adsorption isotherms of human IgM, human IgG, and rat IgG using the membrane Ultrabind TG19318, in different buffers (Castilho, 2001). q*, equilibrium concentration of Ig adsorbed onto the membrane; c*, equilibrium concentration of Ig in the liquid phase.

One of the major limitations in chromatographic processes carried out in packed columns with porous resins is the slow solute diffusion within the particles. The limiting step is the diffusion of the solute towards the immobilized ligands located within the pores, which implies the use of relatively low flow rates (Brandt *et al.*, 1988).

The use of microporous membranes as a chromatography matrix avoids intraparticle diffusional limitations, since their pores, around two orders of magnitude larger than those of conventional resins, are accessed mainly by convection (*Figure 12.10*). This enables the operation at relatively high flow rates, with relatively low pressure drops. Additionally, membrane adsorbers present better mechanical resistance than gels, with no deformation and bed compaction problems. Also, the systems are usually modular and easy to scale up (Klein, 2000; Bueno and Miranda, 2005).

One of the major advantages of membrane chromatography, similarly to EBA, is the possibility for direct processing cell suspensions, enabling an integration of the clarification and primary purification steps. However, this is only possible by the use of membrane modules with suitable hydrodynamics, capable of effectively avoiding membrane fouling (Belford, 1988; Castilho and Anspach, 2003).

Examples of integrated processes using affinity membranes are available in the literature. Vogel *et al.* (2002) purified recombinant human tissue plasminogen activator (rhtPA) directly from a CHO cell suspension, using L-lysine affinity membranes, achieving a yield of 86% and a removal of 95% of contaminant proteins. Castilho *et al.* (2002b) proposed the use of protein A affinity membranes for an integrated perfusion process with simultaneous primary product purification. CHO cells producing an anti-HIV mAb were cultivated in a perfusion bioreactor coupled to a rotating disk filter containing affinity membranes, which promoted not only separation and recycling of the cells to the bioreactor, but also product adsorption. Through periodic elution and regeneration cycles, the authors obtained, in only one step, a product with high purity and 14-fold more concentrated than in the bioreactor (Castilho *et al.*, 2002b).

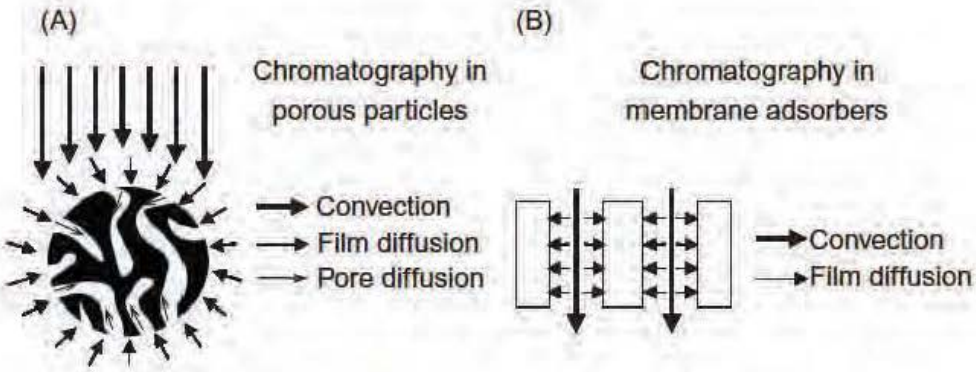


Figure 12.10

Comparison of solute transport to the adsorption sites: (A) in conventional porous resins and (B) in membrane adsorbers. Adapted from Ghosh (2002).

12.5 Conclusions

Figure 12.11 shows the methods most commonly used as consecutive steps in the downstream processing of bioproducts. Precipitation and ion exchange chromatography are commonly employed as the first step, whereas, for such processes composed of five steps, molecular exclusion chromatography is used predominantly as the last step. As shown in *Figure 12.12*, affinity chromatography is the technique with the highest purification factor, followed by hydrophobic interaction chromatography. Despite being frequently used, precipitation has low selectivity and provides a low purification factor.

Downstream processing has an important impact on the final product cost, in same cases reaching 90% of the total production costs (Cunha *et al.*, 2003). There are many reasons for this, and one of them is the high number of steps usually needed to achieve the required purity. Due to an inherent product loss in every step, the global yield of the target protein is usually low. The implementation of redundant steps for facilitating process validation is an additional reason for the high costs of downstream processing.

Therefore, in spite of the large availability of methods for the recovery and purification of proteins, there is still a need for studies in this area, aiming to improve the existing techniques and to develop novel methods to satisfy new demands. The optimization of the recovery and purification process for a given bioproduct may involve conflicting objectives and, therefore, a careful balance should be made of the advantages and

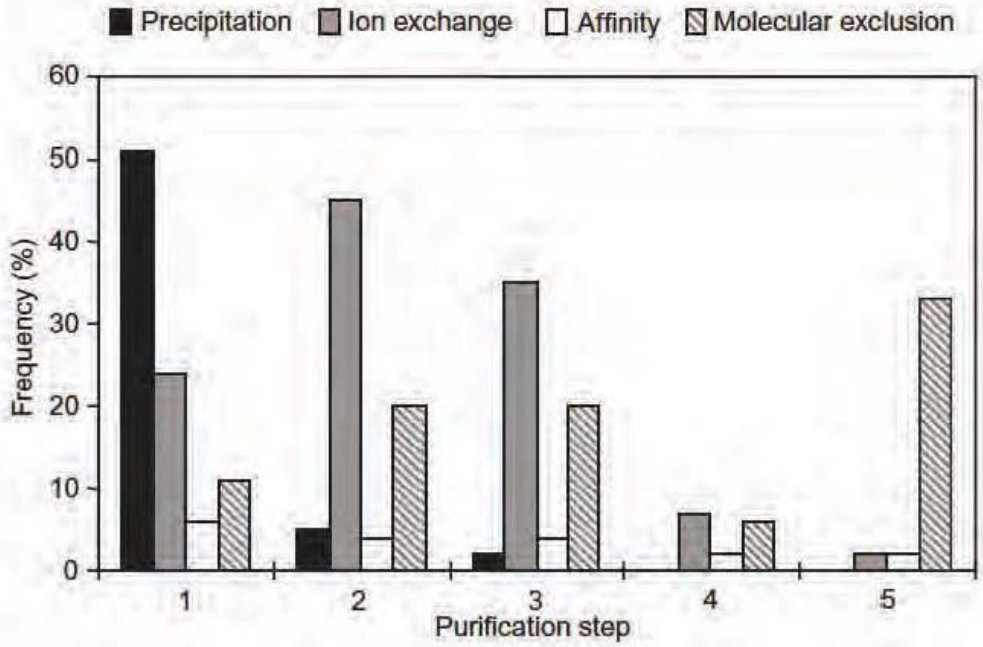


Figure 12.11

Frequency of use of different purification methods in processes composed of five purification steps. Estimates are based on the analysis of 100 articles in international scientific journals published from January 1992 to January 1994 (adapted from Freitag and Horváth, 1996).

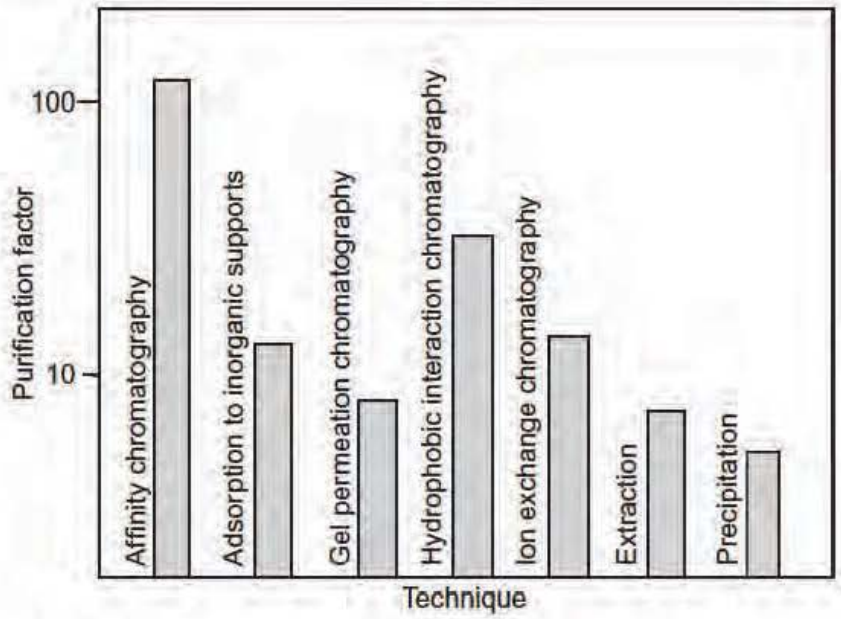


Figure 12.12

Average purification factors achieved using different purification techniques (adapted from Bonnerjea *et al.*, 1986).

disadvantages of every change/innovation proposed for a process. More extensive discussions on the topic presented in this chapter are available in Janson and Rydén (1998), Ladisch (2001), Harrison *et al.* (2003), Pessoa Junior and Kilikian (2005), Scopes (1994), and Harris and Angal (1989).

References

Ameskamp N, Priesner C, Lehmann J, Lütkemeyer D (1999), Pilot scale recovery of monoclonal antibodies by expanded bed ion exchange adsorption, Bioseparation 8:169 188.

Anspach FB, Curbelo D, Hartmann R, Garke G, Deckwer WD (1999), Expanded bed chromatography in primary protein purification, J. Chromatogr. A 865:129 144.

Atkins E, Zamora S, Candia BJ, Baca A, Orlando RA (2005), Development of a mammalian suspension culture for expression of active recombinant human urokinase type plasminogen activator, Cytotechnology 49:25 37.

Belford G (1988), Membrane modules: comparison of different configurations using fluid mechanics, J. Membrane Sci. 35:245 270.

Bonnerjea J, Oh S, Hoare M, Dunnill P (1986), Protein purification: the right step at the right time, BioTechnol. 4:954 958.

Brandt S, Golfe RA, Kessler SB, O'Connor JL, Zale SE (1988), Membrane based affinity technology for commercial scale purifications, BioTechnol. 6:779 782.

Bueno SMA, Miranda EA (2005), Membranas adsortivas, In: Pessoa Júnior A, Kilikian BV (Eds), Purificação de Produtos Biotecnológicos, Ed. Manole, São Paulo, Brazil, pp. 176 195.

Castilho LR (2001), Development of a dynamic filter for integrated perfusion cultiva tion and purification of recombinant proteins from mammalian cells, Fortschritt Berichte Series Reihe 17 (Biotechnologie/Medizintechnik), VDI Verlag, Düssel dorf.

Castilho LR, Anspach FB (2003), CFD aided design of a dynamic filter for mammalian cell separation, Biotechnol. Bioeng. 83:514 519.
Castilho LR, Anspach FB, Deckwer W D (2002a), Comparison of affinity membranes for the purification of immunoglobulins, J. Membrane Sci. 207:253 259.
Castilho LR, Anspach FB, Deckwer W D (2002b), An integrated process for mamma lian cell perfusion cultivation and product purification using a dynamic filter. Biotechnol. Prog. 18:776 781.
Charcosset C (1999), Purification of proteins by membrane chromatography, J. Chem. Technol. Biotechnol. 71:95 110.
Cunha MT, Aires Barros MR, Cabral JMS (2003), Extraction for rapid protein isolation, In: Hatti Kaul R, Mattiasson B (Eds), Isolation and Purification of Proteins, Marcel Dekker, New York, pp. 321 372.
Deml L, Schirmbeck R, Reimann J, Wolf H, Wagner R (1999), Purification and characterization of hepatitis B virus surface antigen particles produced in *Droso phila Schneider* 2 cells, J. Virol. Methods 79:205 217.
El Kak A, Vijayalakshmi MA (1991), Study of the separation of mouse monoclonal antibodies by pseudobioaffinity chromatography using matrix linked histidine and histamine, J. Chromatogr. 570:29 41.
Eriksson KO (1998), Hydrophobic interaction chromatography, In: Janson JC, Rydén L (Eds), Protein Purification: Principles, High Resolution Methods, and Applica tions, 2nd Ed., Wiley VCH, Weinheim, pp. 283 310.
Ersson B, Rydén L, Janson JC (1998), Introduction to protein purification, In: Janson JC, Rydén L (Eds), Protein Purification: Principles, High Resolution Methods, and Applications, 2nd Ed., Wiley VCH, Weinheim, pp. 3 40.
Franco TT, Kilikian BV, Coimbra JSR, Meirelles AJA (2005), Extração líquido líquido em sistemas de duas fases aquosas, In: Pessoa Júnior A, Kilikian BV (Eds), Purificação de Produtos Biotecnológicos, Ed. Manole, São Paulo, Brazil, pp. 114 165.
Freitag R, Horváth C (1996), Chromatography in the downstream processing of biotechnological products, Adv. Biochem. Eng. Biotechnol. 53:17 59.
GE Healthcare/Amersham Biosciences (2000), Gentle STREAMLINE suits fragile CHO cells, Downstream 32:17 23.
GE Healthcare/Amersham Biosciences (2002), Expanded bed adsorption: Streamline 50, 100 and 200 columns. Available at: http://www5.amershambiosciences.com/aptrix/upp00919.nsf/(FileDownload)?OpenAgent&docid=4DA5095795C3BDE1C1256EB40044A9BC&file=18112735AB.pdf
Ghosh R (2002), Protein separation using membrane chromatography: opportunities and challenges, J. Chromatogr. A 952:13 27.
Hahn R, Shimahara K, Steindl F, Jungbauer A (2006), Comparison of protein A affinity sorbents III. Life time study, J. Chromatogr. A 1102: 224 231.
Harris ELV, Angal S (1989), Protein Purification Methods: A Practical Approach, IRL Press, Oxford.
Harrison RG, Todd P, Rudge SR, Petrides DP (2003), Bioseparations Science and Engineering, Oxford University Press, New York.
Haupt K, Bueno SMA (2000), Affinity separation: affinity membranes, In: Wilson ID, Adlard ER, Poole C, Cooke M (Eds), Encyclopaedia of Separation Science, Academic Press, pp. 229 235.
Hearn MTW (1998), High resolution reversed phase chromatography, In: Janson JC, Rydén L (Eds), Protein Purification: Principles, High Resolution Methods, and Applications, 2nd Ed., Wiley VCH, Weinheim, pp. 239 282.
Ho CC, Zydney AL (2000), A combined pore blockage and cake filtration model for protein fouling during microfiltration, J. Colloid Interface Sci. 232:389 399.
Janson JC, Rydén L (1998), Protein Purification: Principles, High Resolution Meth ods, and Applications, 2nd Ed., Wiley VCH, Weinheim.

Johansson G (1998), Affinity partitioning of proteins using aqueous two phase systems, In: Janson JC, Rydén L (Eds), Protein Purification: Principles, High Resolution Methods, and Applications, 2nd Ed., Wiley VCH, Weinheim, pp. 443 460.

Karlsson E, Rydén L, Brewer J (1998), Ion exchange chromatography, In: Janson JC, Rydén L (Eds), Protein Purification: Principles, High Resolution Methods, and Applications, 2nd Ed., Wiley VCH, Weinheim, pp. 145 206.

Kilikian BV, Santos ES (2005), Cromatografia: Adsorção em Leito Expandido (ALE), In: Pessoa Júnior A, Kilikian BV (Eds), Purificação de Produtos Biotecnológicos, Editora Manole, São Paulo, Brazil, pp. 268 279.

Klein E (2000), Affinity membranes: a 10 year review, J. Membr. Sci. 179:1 27.

Kumar A, Galaev IY, Mattiasson B (2003), Precipitation of proteins: nonspecific and specific, In: Hatti Kaul R, Mattiasson B (Eds), Isolation and Purification of Proteins, Marcel Dekker, New York, pp. 225 276.

Kumar A, Bansal V, Andersson J, Roychoudhury PK, Mattiasson B (2006), Super macroporous cryogel matrix for integrated protein isolation immobilized metal affinity chromatographic purification of urokinase from cell culture broth of a human kidney cell line, J. Chromatogr. A 1103:35 42.

Kumpalume P, Ghose S (2003), Chromatography: the high resolution technique for protein separation, In: Hatti Kaul R, Mattiasson B (Eds), Isolation and Purifica tion of Proteins, Marcel Dekker, New York, pp. 29 56.

Låås T (1998), Electrophoresis in gels, In: Janson JC, Rydén L (Eds), Protein Purifica tion: Principles, High Resolution Methods, and Applications, 2nd Ed., Wiley VCH, Weinheim, pp. 463 494.

Ladisch, MR (2001), Bioseparations Engineering: Principles, Practice, and Economics, John Wiley and Sons, New York.

Lali A, Balan S, John R, D'Souza F (1998), Carboxymethyl cellulose as a new heterobifunctional ligand carrier for affinity precipitation of proteins, Biosepara tion 7:195 205.

Maugeri Filho F, Mendieta Taboada O (2005), Cromatografia de Interação Hidrofóbi ca, In: Pessoa Júnior A, Kilikian BV (Eds), Purificação de Produtos Biotecnológi cos, Ed. Manole, São Paulo, Brazil, pp. 212 230.

Moraes AM, Vieira e Rosa PT (2005), Cromatografia de exclusão molecular, In: Pessoa Júnior A, Kilikian BV (Eds), Purificação de Produtos Biotecnológicos, Ed. Manole, São Paulo, Brazil, pp. 176 195.

Pessoa Júnior A, Kilikian BV (2005), Purificação de Produtos Biotecnológicos, Ed. Manole, São Paulo, Brazil.

Rasmussen LK, Larsen YB, Hojrup P (2005), Characterization of different cell culture media for expression of recombinant antibodies in mammalian cells: presence of contaminating bovine antibodies, Protein Expres. Purif. 41:373 377.

Romero J, Zydney AL (2002), Affinity ultrafiltration: effects of ligand binding on selectivity and process optimization, Biotechnol. Bioeng. 77:256 265.

Roper DK, Lightfoot EN (1995), Separation of biomolecules using adsorptive mem branes, J. Chromatogr. 702:3 26.

Scheich C, Sievert V, Büssow K (2003), An automated method for high throughput protein purification applied to a comparison of His tag and GST tag affinity chromatography, BMC Biotechnol. 3:12 19.

Scopes RK (1994), Protein Purification: Principles and Practice, 3rd Ed., Springer Verlag, New York.

Serpa G, Augusto EFP, Tamashiro WMSC, Ribeiro MB, Miranda EA, Bueno SMA (2005), Evaluation of immobilized metal membrane affinity chromatography for purification of IgG1 monoclonal antibody, J. Chromatogr. B 816:259 268.

Thömmes J, Kula MR (1995), Membrane chromatography an integrative concept in the downstream processing of proteins, Biotechnol. Progr. 11:357 367.

Vijayalakshmi MA (1989), Pseudobiospecific ligand affinity chromatography, Trends Biotechnol. 7:71 76.
Vijayalakshmi MA (2002), Biochromatography; Theory and Practice, CRC Press, New York.
Voet D, Voet JG (1995), Biochemistry, 2nd Ed., John Wiley & Sons, New York.
Vogel JH, Anspach FB, Kroner KH, Piret JMM, Haynes CA (2002), Controlled shear affinity filtration (CSAF): a new technology for integration of cell separation and protein isolation from mammalian cell cultures, Biotechnol. Bioeng. 78:806 814.
Walsh G, Headon DR (1994), Protein Biotechnology, John Wiley & Sons, West Sussex.
Wheelwright SM (1991), Protein Purification: Design and Scale Up of Downstream Processing, Hanser Publishers, New York.

Quality control of biotechnological products

13

Marina Etcheverrigaray and Ricardo Kratje

13.1 Introduction

Within the pharmaceutical industry, the term biotechnology, in its broadest definition, refers to the use of live organisms to produce biomedicines. The general regulatory scheme for biotechnological products is the same as for products made by traditional methods, with additional specific requirements based on their biological origin. The aim of this chapter is to consider the criteria for the production and quality control of products obtained through biotechnological techniques. In Chapter 14 (Regulatory aspects), complementary information is supplied about controls required by governmental organizations for product registration and quality guarantees, during a product's lifetime in the market. These controls are part of a set of regulations and guidelines known as Good Manufacturing Practice (GMP).

The main difference between traditional and biotechnological pharmaceutical products lies in the fact that the latter are produced from live organisms that are often genetically modified. This category includes not only proteins or polypeptides derived from recombinant DNA (rDNA) but also monoclonal antibodies (mAbs). In the production stages, all the pharmaceutical products, even those of biotechnological origin, share the same basic requirements for process validation, environmental control, aseptic production, and quality control systems. However, the biotechnological systems usually present a greater degree of complexity.

The rDNA-derived products may contain potentially harmful contaminants that are normally not present in their equivalents prepared by chemical methods, and which the purification process must be capable of eliminating, such as the endotoxins expressed in bacterial cells, cellular DNA, and viruses of animal origin. Contamination with nucleic acid from transformed mammalian cells is of particular concern due to the possible presence of potentially oncogenic DNA.

The selected production procedure affects the nature and level of potential contaminants. The possible variability of the system during production may result in modifications that favor the expression of alternative genes in the host–vector system or produce lower product efficiency, or quantitative and qualitative differences in the present impurities. An example of such variability is a change in the profile of proteases

and this should be evaluated, as this may affect the quality of the recombinant protein produced (Kratje *et al*., 1994). The production of continuous cultures is subjected to similar considerations. Therefore, it is important to rely on procedures that assure the uniformity of the conditions of production and consequently the uniformity of the final product.

The development of biological materials as therapeutic agents must involve guarantees about their safety, efficacy, and quality. Safety and efficacy are established through well controlled toxicity studies and clinical trials. Biological tests are important as a direct measure of biological activity and as safety indicators that may detect potential deviations related to efficacy and safety. Quality should be confirmed using a variety of analytical techniques, which can evaluate purity, biological potency, stability, and consistency of production.

With the aim of establishing lot-to-lot consistency, the characterization of a target protein should be performed during its development, to establish the required analytical criteria for production. This characterization may be continued during its manufacture. The evaluation of a product is based on its adequate characterization before and during process development. This involves exhaustive research on the structure, physicochemical properties, and biological activity, as well as its potential contaminants (Jeffcoate *et al*., 1993). For a full overall evaluation, it is necessary to use a combination of these procedures, as none of these can provide enough information on their own.

Biotechnological production processes may include the following stages.

(i) Stage of cell expansion (Upstream). This consists of obtaining sufficient producer biomass or cells by bacterial fermentation or cell culture. After this an impure biomolecule may be obtained under conditions of pH, ionic strength, aggregation state, etc., which must be optimized during subsequent processing.
(ii) Purification (Downstream). At this stage a molecule with the required degree of purity is produced from the crude culture material while fully maintaining its biological and therapeutic activity. This process must ensure that the product is treated with criteria acceptable for its eventual use as a pharmacological agent. The resulting material may be called pure raw material (active pharmaceutical ingredient).
(iii) Galenic conditioning. The pure raw material must be conditioned to achieve a final galenic formulation fulfilling all the requirements of a pharmaceutical product, such as suitable pharmokinetics and safety.

The present chapter includes the following applications of cell cultures:

(i) **Production of recombinant proteins** (WHO, 1991),
   – the initial materials, including the basic data of the host cell and the origin, nature, and sequence of the gene used in production;
   – its production process;
   – the pure raw material;

(ii) **Bioassays** (Mire-Sluis *et al*., 1996; Mire-Sluis and Thorpe, 1998).
   – Special emphasis on bioassays performed in cell culture.

rDNA-derived products and mAbs are considered to be similar to biological substances produced by traditional methods, such as bacterial and viral vaccines, in which adequate control of the initial materials and the production procedure is as necessary as that of the product itself. These considerations, therefore, put considerable emphasis on control during the production, to insure the product's safety and efficacy.

As detailed in Chapter 14 (Regulatory aspects), both the production process validation and purification capacity to eliminate undesirable materials are considered essential. The general criteria for quality control of biotechnological products involve special attention to the quality of all components used in production, including the components of fermentation and culture media. If additives of animal origin are used (e.g. fetal calf serum), they should be proved to be free from adventitious agents. It is not acceptable to use any agent with potential to provoke allergic reactions, such as penicillin or other beta-lactam antibiotics.

Biotechnological products must satisfy the general criteria for the quality control of biological products, such as tests of activity, abnormal toxicity, pyrogenicity, stability, and sterility. The chemical identity of the target molecule must also be assured, comparing it to a reference, and a group of tests that verify its integrity, degree of aggregation, and correct amino acid sequence.

As well as these general criteria there may be specific tests appropriate to specific products with regard to quality control. Therefore, each product's production and quality control should be considered independently, taking into account any special characteristic. The considerations for each product must also relate to its clinical use. Therefore, a preparation to be administered repeatedly over a long period of time or in large doses will probably need to be subjected to more stringent tests (particularly for antigenicity) than a product that is applied only once (e.g. a vaccine).

It is important to determine permissible maximum limits for impurities and contaminants that may be present in these products, adapting the limits according to technological advances for testing or from the continuous assessment of the effects of clinical applications.

13.2 Production of recombinant proteins

13.2.1 Control of starting materials

Cloning and expression

As detailed in Chapter 14, regulatory organizations require information on:

(i) Host cell characterization. This includes origin, phenotype and genotype, and the description of the culture medium, morphological aspects, and growth rate. For eukaryotic cells used as host, the cell

line history and general characteristics should be provided. These must be stable from the master cell bank to the end of production. If cells have a finite lifespan, the maximum number of population doublings should be determined (CBER, 1993).

(ii) Characterization of host–vector system. Documentation concerning the gene cloning strategy and characterization of the recombinant vector is required (International Conference of Harmonization Guidelines, 1995).

Cell banks

Once the cell line is selected as a host for production, a cell bank system must be generated to guarantee the availability of an adequate source of equivalent cells to use through the product's lifetime in the market. Usually, the cell bank system consists of two levels: a master cell bank (MCB) and a manufacturer's working cell bank (WCB).

Master cell bank (MCB)

This is a homogeneous suspension of the original cells, already transformed with the expression vector and containing the desired gene, which is distributed in equal volumes into individual containers (ampoules) for storage (usually in liquid nitrogen). In some cases, it may be necessary to establish a separate MCB for the expression vector and the host cell. The localization, identification, and inventory of the individual ampoules must be carefully documented.

All the necessary MCB identity tests should be performed to establish the most significant cell characteristics and their stability over time. Data must be provided to prove that the cells can be used for the desired aim, as well as data showing the gene copy number and identity of the expression system, and the quantity and quality of the protein being produced. Normally, the nucleotide sequence of the cloned gene in the MCB should be confirmed. However, in certain cases, as when multiple copies of the cloned gene are introduced into the genome of the host cell, sequencing can be inappropriate. In these circumstances, it might be useful to perform either a Southern blot analysis of the total cellular DNA (Southern, 1975; Chaplin and Kennedy, 1994) or a Northern blot from the mRNA sequence (Alwine *et al*., 1977). In these cases, particular attention must be paid to the characterization of the final product (see Section 13.4).

If a clone is selected from a population of host cells into which an expression construct has been transferred, it will be necessary to create a new MCB. From this MCB it will be important to guarantee the identity of the clone and the produced protein in reference to the original.

Working cell bank (WCB)

This is derived from a homogeneous suspension of cells derived from the MCB after a finite number of passages, distributed in equal volumes in ampoules for storage. The localization, identification, and inventory of the individual ampoule must be carefully documented.

For the production of a lot of biological product, one or more WCB ampoules can be used. If cells from more than one ampoule are used, the

cellular suspensions must be mixed immediately after thawing. The population doubling number of the cells used for production will be based on previous criteria established by the manufacturer, assuring the integrity of the cell product system. A WCB can be used for the production of cultures with a finite number of passages or continuous cultures.

In both banks:

(i) All ampoules must be treated equally during storage, and once removed from storage they must be discarded;
(ii) It is recommended that each MCB and WCB should be stored in two or more sufficiently separated areas inside the production facilities to avoid loss of the cell line.

13.2.2 Quality control of cell banks

The quality control of cell banks must include information on the following.

(i) Stability by measurements of vector viability and retention.
(ii) Cellular identity through phenotypic characterization.
(iii) Evidence must be presented, proving that cell banks are free from potential infectious or oncogenic agents (viruses, bacteria, fungi, or mycoplasma). Special attention should be paid to viruses that commonly infect the species from which the cell line is derived. Certain cell lines contain endogenous viruses, for example, retrovirus, whose elimination might not be feasible. The expression of these endogenous viruses should be tested in a variety of conditions that may cause their induction. It is important to show that all lots of cells are free from every adventitious contaminant.
(iv) In the case of eukaryotic cells derived from mammals, information concerning the potential tumorigenicity of the cell bank is necessary.
(v) Accurate records must be maintained of the composition and origin of the culture medium used for the cell banks. As animal based sera can produce allergic reactions in human beings, efforts must be made to significantly reduce the level of serum required for the propagation and production in cell cultures. The residual quantity of serum or additives in the final product must be determined and it must not exceed the established limits.

If porcine trypsin is used in the passage of cells, it should be free from adventitious agents, including porcine parvovirus. The manufacturers of biological products must provide information about the source(s) and controls of any material derived from ovine or bovine species.

Penicillin or other beta-lactam antibiotics should not be used in production cell cultures. Minimum concentrations of other antibiotics or inductor agents in the culture may be acceptable; however, the presence of any antibiotic or inductor agents in the final product is not acceptable.

13.3 Control of the production process

13.3.1 Cultures

Cultures with a finite number of passages

This culture method is defined by a limited number of passages or population doublings, which should not be surpassed during the production process. The maximum number of doublings or passages should be established for the process.

The procedure and materials used for cell propagation and product induction should be described in detail (see Chapter 14). For each production lot, data should be given about the scope and nature of any microbial contamination of culture containers immediately before collection. The sensitivity of the methods used for detection should be described.

Data should be available about the uniformity of the fermentation conditions and cell propagation, and about the maintenance of the product yield (cell concentration and viability, nutrient and metabolite concentrations, product concentration, etc.). The criteria as to when to discard a culture should be established (when they are not included in the uniformity specifications mentioned). The characteristics of the host cell and vector at the end of production cycles should be observed. If pertinent, the nucleotide sequence of the insert coding the cloned DNA-derived product should be determined at least once after the culture is carried out on a large scale.

Continuous cultures

The number of population doublings or passages in this type of culture is neither defined nor restricted for production. The manufacturer will define the criteria adopted for both the cell culture and the end of the production process (see above – Cultures with a finite number of passages). During the culture stage, these criteria must be monitored; the frequency and type of monitoring will depend on the nature of the production system and the product (Bollati *et al*., 2005). These should be defined and recorded when the product is registered.

Information should also be given about the integrity of the gene that is being expressed and the phenotypic and genotypic characteristics of the host cell after a long term culture. If pertinent, the nucleotide sequence of the insert that codes the cloned DNA-derived product should be determined, at least once after a large scale culture. Although, as mentioned in the section on the MCB above, when multiple copies of the cloned gene are inserted in the genome of a continuous cellular line, it may be inappropriate to sequence the cloned gene.

Data should also be presented showing that the variations in cell density and product concentration fall within established limits. The acceptance of culture supernatants for further processing must be clearly linked to the predefined program adopted, and this will depend upon a clear definition of the characteristics of a ‘‘product lot.’’ The criteria to discard suspensions and stop cultures, when they do not follow the specifications (see

above), should also be established. Systemic tests should be performed to investigate the microbial contamination according to a pre-defined harvesting strategy.

The maximum length of a continuous culture should be based on information about the system and product uniformity and stability. In long continuous cultures, the cell line and product will be repeatedly evaluated at intervals determined by the information on the stability of the host–vector system and the product characteristics.

13.3.2 Purification

The methods used in the recovery, extraction, and purification must be described in detail. Special attention must be paid to the elimination of viruses, nucleic acids, and undesirable antigenic materials.

In procedures involving affinity chromatography, which may use biological components such as mAbs, appropriate measures should be taken to ensure that no contamination arises from its use, such as adventitious viruses, that could threaten the safety of the final product. The more commonly used methodologies to determine the levels of contamination are presented in Section 13.4.8.

The ability of the purification process to eliminate product related or host cell derived proteins, nucleic acid, carbohydrates, viruses, or other undesirable impurities, including undesirable media derived and chemical components, must be thoroughly investigated, as well as the reproducibility of the process.

13.4 Product control

13.4.1 Characterization and specification

The requirements for identity, purity, activity, and stability of the product are closely related to the processing technology and the physicochemical and biological characteristics of a specific drug. The planned use of the product should also be considered.

In general the quality control procedures for products obtained through biotechnology are very similar to those routinely used with traditional pharmaceutical products in areas such as raw material testing, documentation of process control, and aseptic processing. The fundamental difference is in the type of methods used, so as to determine the product's identity, uniformity, and purity. In the quality control of products obtained through recombinant DNA technology, it is necessary to employ validated tests for the final and intermediary products to ensure the elimination of undesirable impurities.

It is essential to characterize the final active substance through chemical, physical, and biological methods. Special consideration should be given to the use of a range of analytical techniques to determine a range of physicochemical properties of the molecule. The methods used must be validated and should have a known sensitivity.

Contamination of the product generally may come from three sources:

(i) host organism: proteins or DNA of the host;
(ii) proteins and impurities from the production process – mainly from the purification stage;
(iii) impurities related to the active substance, for example, aggregation state, reduced or oxidized forms.

The purity of a protein preparation obtained through recombinant DNA technology should be maximized. The allowed levels of contaminants for each product should be specified for the manufacturing process.

13.4.2 Protein content

Quantification of total protein is very important because the values of other product parameters depend on the accurate determination of protein content, for example the specific activity.

There are various accepted methods to determine the protein content, namely:

(i) **UV absorbance.** This is one of the simplest methods and it does not require a standard. However, the coefficient of molar extinction of the protein should be known because it is specific for each protein (Goldfarb *et al*., 1951).
(ii) **Lowry method.** This is a colorimetric method and needs to be compared to a standard, e.g. the bovine serum albumin (Lowry *et al*., 1951). The reaction products are spectrophotometrically quantified between 540 and 560 nm. This technique is linear at the microgram range and can be used for any protein. Alcohol, sugars, and detergents interfere in the measurements, making their removal necessary before any reliable measurement.
(iii) **Bradford method.** This is a colorimetric method that uses Coomassie brilliant blue, which forms a conjugate with proteins under acidic conditions (Bradford, 1976). An unknown needs to be compared to a standard, e.g. bovine serum albumin. The reaction products are quantified spectrophotometrically at 595 nm. The technique is linear at the microgram range and can be used for any protein. There are various substances/factors that interfere with the measurements (SDS, Tween, Triton-X100, high ionic strength, alkaline pH) making their removal necessary before any reliable measurement.
(iv) **Kjeldahl method.** This is a technique used to determine the amount of nitrogen present in a protein. It is estimated that 1 mg of nitrogen equates to 6.5 mg of protein (Kjeldahl, 1883).

13.4.3 Amino acid analysis (identification and/or protein content)

The method consists of the complete hydrolysis of a protein or peptide to release its component amino acids. These may be separated by reverse phase high performance liquid chromatography (HPLC), and quantified with fluorometric detection after derivatization with *o*-phthaldehyde (Larsen and West, 1981). The method serves to determine both the amino acid composition and the total quantity of protein present in the sample.

Disadvantages of this method include the total or partial destruction of some amino acids (e.g. tryptophan, serine, and threonine), the underestimation of the quantity of amino acids that are difficult to hydrolyze (valine and isoleucine), and the difficulty of quantifying cysteine and methionine, except by prior oxidation.

The amino acid composition should be determined from the average of a minimum of three separate hydrolyses per lot.

13.4.4 Protein sequencing (identification)

This technique gives information about the protein's primary structure, which may include its amino and/or carboxyl terminal groups (Edman, 1950). For recombinant DNA-derived proteins, this analysis serves to confirm the amino acid sequence predicted by the DNA sequence. The analysis can also be useful to determine the protein's homogeneity.

(i) Amino-terminal: This is a classic chemical technique of sequential breakdown from the N-terminal end of a protein and can provide limited, but rapid, sequence information to provide data on homogeneity and identity. The method is semi-quantitative, and the data cannot be used to determine the exact proportions of amino acids originating from different N-terminals if present in a protein.
(ii) Carboxyl-terminal: This provides sequence information about the primary structure by degradation from the C-terminal end. The method is also semi-quantitative.

13.4.5 Peptide mapping

This method consists of specific cleavage of the target protein using an endoprotease or by a chemical method. The resulting peptides are separated by reverse phase HPLC or ionic exchange to obtain a highly specific profile.

Peptide mapping is a method that enables the determination of protein identity when compared to a standard. When compared to previous lots of the same product, it serves to determine the stability of the protein's primary sequence, which in turn reflects the genetic stability of the producer cells. This method is capable of detecting small differences between proteins in one or more amino acids. The detection will be dependent upon an amino acid alteration affecting the observed peptide profile (*Figure 13.1*; see color section).

13.4.6 Electrophoresis

Electrophoresis methods are among the most common and potent used in the evaluation of protein purity and homogeneity. They are valuable indicators of protein stability because they detect small molecular or chemical changes in the product caused by denaturation, aggregation, oxidation, deamidation, etc. One of the advantages is that they require only microgram amounts of a sample.

The two most widely used types of electrophoresis tests are: (i) sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE); and (ii) isoelectric focusing (IEF).

SDS-PAGE

This test separates proteins according to their molecular weight (Laemmli, 1970). Firstly, the sample is denatured in a detergent, which breaks the non-covalent intra- and inter-molecular links of the proteins, and then it is separated electrophoretically by a polyacrylamide gel. The electrophoretic separation must be performed under reduced and non-reduced conditions to determine the existence of impurities with the same molecular weight.

The method under non-reduced conditions is normally used to estimate the aggregation and/or oligomerization of the protein but only aggregates or oligomers that are stable in the presence of SDS will be detected and under the conditions used for the sample preparation and electrophoresis.

In the method validation, low concentrations of the product are used for the detection of the target protein, while high concentrations are recommended for the detection of impurities.

The sample detection after electrophoresis can be quantified by a densitometric analysis of Coomassie brilliant blue stained spots. A silver stain is available to provide higher sensitivities when detection of samples in the nanogram range is required.

SDS-PAGE, together with the Coomassie brilliant blue coloration, is used to quantitatively determine the sample purity with respect to dimers, larger covalent aggregates, and polypeptide fragments. The SDS-PAGE protein separation can be combined with the Western blot analysis, which is used to determine the presence of an electrophoretic band (*Figure 13.2*; see color section).

To calculate the percentage purity from a dilution series, the lane in which the protein of interest is no longer visible is determined (Lm). Similarly, for each band of impurity, the lane in which it is no longer visible is determined (Li).

$$\mathrm{I}(\%) = \frac{\mathrm{Pm}}{\mathrm{Pi}} \times 100$$

where *I (%)* is the estimated percentage of impurity, *Pm* the minor protein mass applied which can be visualized and which corresponds to lane Lm-1 and *Pi* is the protein mass applied in lane Li-1. The sample's percentage purity is calculated by adding the impurity percentages corresponding to the different impurity bands and deducting this result from 100. In order to apply this principle it is assumed that there are no impurity bands at the same position as the target protein.

The SDS-PAGE protein separation can be combined with Western blot analysis, which is used to detect the band of the target protein.

After electrophoresis, proteins are transferred onto a nitrocellulose or PVDF (polyvinylidene fluoride) membrane, which is immersed in a solution of a selected antibody capable of combining with the target protein. Then the reaction of antigen-specific antibody is detected through a second antibody which is tagged enzymatically. Special attention must

be paid to impurities of the immunogen used in production of the antibody (Amadeo *et al*., 2004; Oggero *et al*., 2006).

Isoelectric focusing (IEF)

This separates proteins according to their charge in an electric field (Svensson, 1961). The protein charges result from its amino acid composition and the presence of charged molecules from post-transductional modification (e.g. sialic acid molecules in complex glycan structures derived by glycosylation). However, there is a specific pH (the isoelectric point, pI) for each protein, in which the opposite charges on each protein molecule balance, providing a net charge of zero.

The IEF separation is performed with native proteins in polyacrylamide with a large pore size or with agarose gel with ampholytes (amphoteric ions of low molecular weight), which establish a pH gradient due to their migration inside the polymer matrix when an electric field is applied. In the presence of an electric field, sample proteins with a positive charge migrate to the cathode and proteins with a negative charge migrate to the anode. The migration ends when each protein reaches the pH value corresponding to its specific pI value. Here, its net charge is zero. As the protein's migration depends on its amino acid composition, the altered forms of proteins or non-target proteins will migrate to different points. The IEF gels can be colored for the detection of bands with Coomassie brilliant blue or silver stain. This technique can also be combined with an immunological method through electro transference of the bands to a nitrocellulose or PVDF membrane, after electrophoresis, and its later reaction with a tagged antibody, either in an enzymatic or radioactive form.

IEF is used as a purity test and for protein characterization, comparing it with the relative position of an authentic band of the target protein. It can also be used as a method to evaluate the stability of a biological product. Small changes such as the deamination of an amino acid will cause a change in the pI and a modification of the band pattern.

The results of the IEF gel can occasionally be difficult to interpret and its applicability should be considered for each protein (*Figure 13.3*; see color section).

High performance capillary electrophoresis (HPCE)

HPCE offers the advantage of high resolution protein analysis (Dolnik, 2006). Through this method it is possible to analyze the content of isoforms recovered after the recombinant protein purification. Each peak corresponds to a single isoform and the determination of the relative area under each peak enables the determination of the percentage content of each isoform. In some cases this can be used an as an assessment of a protein's quality, when the relationship between biological activity and structure is known (Etcheverrigaray *et al*., 2005) (*Figure 13.4*; see color section).

13.4.7 Carbohydrate determination

Glycosylation is a possible post-translational modification, characteristic of recombinant proteins expressed from eukaryotic cell lines (see Chapter 6).

Glycosylation can influence pharmacokinetics and protein function, so changes in the glycosylation profile can have a significant impact on the pharmacodynamic characteristics and consequently therapeutic efficacy (Sinclair and Elliot, 2004; Marini *et al.*, 2005).

The glycosylation profile is affected by the cell culture conditions. Ideally, a product should be characterized at least once to identify the potential glycosylation sites as well as the specific carbohydrate at each site.

The quantification of specific sugars and total carbohydrates should be performed in each lot. In some cases, IEF and capillary electrophoresis can be alternative methods for determining the percentage of each isoform in a product sample lot. It is often important to determine the isoform profile for each lot prior to release. It would be ideal, but not always possible, to relate the isoform profile to the specific activity of the product.

Two main approaches can be taken to determine the sugar covalently linked to the glycoprotein. Microheterogeneity of glycans is a common phenomenon of glycoproteins giving rise to variability in glycoforms between molecules. Therefore the information on carbohydrate content represents either the average composition or representative structures. The first approach is to determine the composition of glycoprotein-linked sugars. The second approach is to release and separate the structures of individual oligosaccharides covalently bound to glycoprotein. This approach serves to obtain an oligosaccharide map similar to the peptide map for a protein (Chaplin and Kennedy, 1994).

More information about this subject can be obtained in Chapter 6 (Post-translational modification of recombinant proteins).

13.4.8 Potential impurities and contaminants of biotechnological products

Table 13.1 describes the most frequent types of impurities and contaminants, indicating the most suitable detection methods as a guide.

The residual host cell DNA may represent a different threat as an impurity for each product, since it depends on the host organism and the purification process. The control of residual DNA is a measure of efficacy and consistency in the purification process, as well as the product quality. The levels of DNA should be quantified using methods of an adequate sensitivity. Among the techniques used, the following can be mentioned.

(i) Slot blot hybridization, using specific probes tagged with radioactive phosphorus (^{32}P). This is the most sensitive and routinely performed measurement (Pepin *et al.*, 1990).
(ii) Biosensors technology. This methodology is more appropriate for the determination of total DNA/nucleic acid impurities than specifically for host cell DNA.
(iii) Polymerase chain reaction technology (PCR). Adequate primers should be used.

Table 13.1 Impurities and contaminants in processes to obtain biotechnological products

Types	Detection method
Impurities	
Endotoxins	Bacterial endotoxins test, pyrogen test
Host cell proteins	SDS PAGE, immune assays
Other protein impurities	SDS PAGE, HPLC, immune assays
DNA	DNA hybridization, ultraviolet spectrophotometry, PCR
Mutant proteins	Peptide mapping, HPLC, IEF, mass spectrophotometry, amino acids sequencing
Formyl methionine	Peptide mapping, HPLC, mass spectrophotometry
Proteolytic cleavage	IEF, SDS PAGE, HPLC, amino acids sequencing
Protein aggregates	SDS PAGE, HPSEC
Deamination	IEF, HPLC, mass spectrophotometry, amino acids sequencing
Monoclonal antibodies	SDS PAGE, immune assays
Amino acids substitution	Amino acids sequencing and analysis, peptide mapping, mass spectrophotometry
Contaminants	
Microbial contaminants (bacteria, yeasts, fungi)	Hygienic control, sterility test, DNA
Mycoplasma	PCR, DNA
Virus (exogenous and endogenous)	CPE, Had (only exogenous virus), reverse transcriptase activity, PMA

SDS PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography; PCR, polymerase chain reaction; IEF, isoelectric focusing; HPSEC, high performance size exclusion chromatography; DNA, DNA binding fluorochrome; CPE, cytopathic effect; Had, hemadsorption; PMA, production of murine antibodies.

13.5 Bioassays

Methods to determine the potential biological activity of products obtained through recombinant DNA techniques are of fundamental importance. Despite the existence of numerous physicochemical techniques to characterize the protein product structure and the presence of contaminants, they provide little, if any, information about its biological potency. A bioassay is defined as a functional test, and no physicochemical test can measure the function. However, for some peptide hormones, which are less complex in structure than most cytokines, well defined physicochemical tests may be used to estimate biological activity; for instance, the capillary electrophoresis analysis of a protein's isoform content if the specific activity of each one is known.

A bioassay is an analytical procedure that uses a responsive biological system (biological function) to measure the biological potency of a product (Mire-Sluis *et al*., 1996). The most appropriate method to determine it is by comparing the biological activity of a sample with a well-characterized reference standard.

The biological activity measured is often expressed in international units, but should be recorded in relation to the product mass. This means that the biological effect is measured as activity per unit of mass and

should be consistent within clearly specified limits. The activity expressed per unit of mass is called specific activity and it constitutes a parameter of identity and/or purity.

However, the bioactivity measured in an *in vitro* assay may not be considered as a direct biological activity indicator in the human. Thus it has been shown that a biological assay for a cytokine does not necessarily reflect the clinical efficacy (Thorpe *et al*., 1997).

13.5.1 Bioassay types

The first bioassay type evaluated the response of cytokines directly in animals. However there are many disadvantages to animal tests including inter-animal variability, their expense, demand for intensive work, and ethical concerns when many animals may have to be sacrificed to provide statistically valid data. Significant variability can result from the sex, age, species, and health of the animals used.

Cell culture technology can be a viable alternative to animal testing in many cases, with the possibility of increasing significantly the number of replicate samples and thus expanding the bioassay utility. Primary cell cultures have the advantage that they maintain most of the characteristics of the animal tissue from which the cells are derived. These would be ideal for bioassays except that they cannot be kept in culture indefinitely. They can be difficult and sometimes impossible to grow reproducibly, and are subject to the inherent variability of the animal they come from.

Consequently, most bioassays for cytokines are performed using established cell lines that can be grown indefinitely. There are many advantages associated with the use of such lines. They are available from frozen cell banks and do not require direct isolation from primary animal tissue. They are generally clonal and so their growth is relatively consistent, allowing relatively consistent results over time. As indicated earlier there is an inherent variability associated with the use of living systems, whether entire organisms, *ex vivo* tissue explants, or isolated cells. However, the use of continuous cell lines would tend to minimize these problems. The cells may lose cell function and suffer genetic changes over extended culture passage. However, this can be controlled by maintaining a large enough cell bank at low passage number for most experts and so any such disadvantage is more than compensated for by their advantages.

Despite the diverse existing varieties of bioassays for cytokines, all are based on the protein's capacity to induce a measurable activity in cells and tissues. The cell responds in various ways including: enhanced growth, growth inhibition, expression of cellular markers, cytotoxicity, or antiviral activity (Wadhwa *et al*., 1995).

The selection of the type of assay depends on the nature of the biological material available for assay. The bioassays can demonstrate the consistency of the product's biological activity by replicate analysis as well as define the potency of each lot. *Figure 13.5* shows the analysis of biological activity of a sample of recombinant human interferon-β1a (rhIFNβ1a).

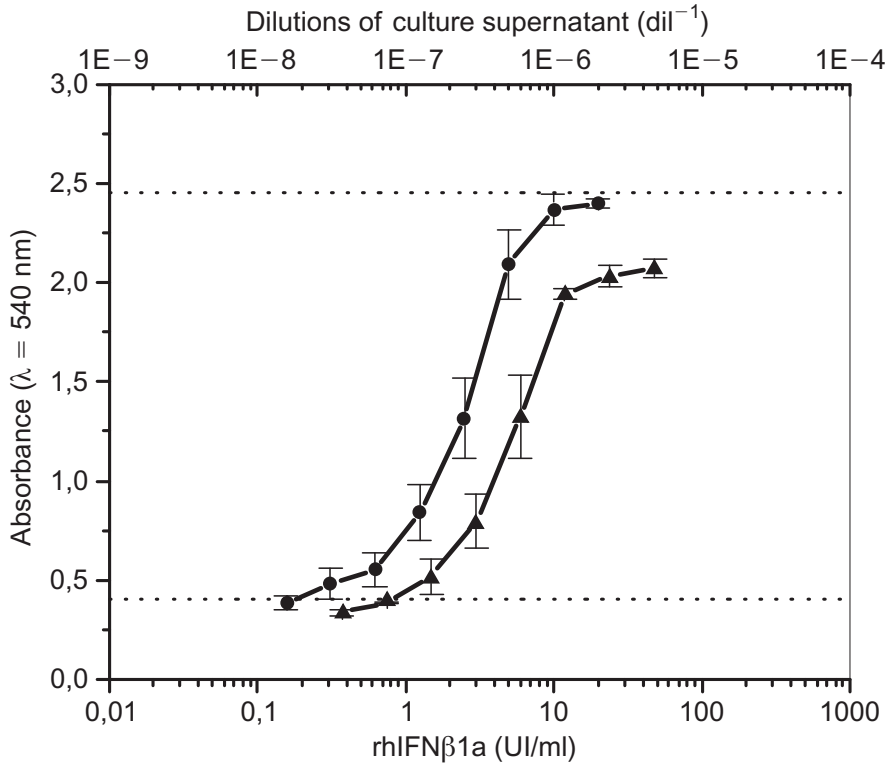


Figure 13.5

Determination of the *in vitro* biological activity of rhIFNβ1a (recombinant human interferon β1a). Standard (●), production sample of rhIFNβ1a (▲). The curves corresponding to the standard and the sample were processed in triplicate and the results are shown as the average value ± SD. The dotted horizontal lines indicate the average values of the test's negative and positive controls processed in quadruplicate.

13.5.2 *In vitro* bioassays

The major advantage of a bioassay is the capacity to detect only functional molecules. However, despite this, there are also important disadvantages. These include the low specificity, which may be attributed to the nature of the molecules themselves, including their pleiotropic actions, their diversity of target cells, and their ability to induce different effects on different cell types. In addition, the same function may be performed by two different cytokines, probably through the same cellular receptor. This is a disadvantage when dealing with samples that may contain multiple cytokines, such as clinical samples (Cannon *et al*., 1993; Whiteside, 1994). However, the inclusion of specific neutralizing antibodies for target proteins may permit the determination of the activity of a single protein within a mixture.

Contamination (including mycoplasma) and differences in serum lots for cultures can affect bioassay performance. Consequently, it is important to maintain cell lines adequately and use consistent sources of material to ensure reproducibility of test results. Bioassay data generally follow a sigmoidal curve, which can complicate the interpretation of results.

The ability to obtain cell clones that respond to cytokines and serve as source material for bioassays has aided the development of bioassays. The cell lines, obtained mainly from human or murine cancers, often depend on a cytokine for their growth and tend to be immortal, thus providing a unique homogeneous cell source, which may be distributed among laboratories (Mire-Sluis and Thorpe, 1998). The advent of recombinant DNA technology has enabled the cloning of specific cytokine receptors and their expression in any cell line (Canosi *et al*., 1996). In this way, a cell line can be created that responds specifically to almost any cytokine, and avoids the necessity to isolate a cell line with an endogenous receptor.

13.5.3 Experimental design

An *in vitro* bioassay can be designed in several ways, but requires statistical validity. A one point assay is not valid. The bioassay should be designed to consider factors that introduce variability, and the analysis should test such variability. A measurement series of a test sample should be compared to an equivalent series of the reference material, carefully considering the comparisons between the linear portions of the dose-response curves (Mire-Sluis *et al*., 1996). To test validity of a bioassay inter- and intra-assay variability should be considered in both preparation, and in the case of multiwell plates, the variability between each plate. To reduce the positional effect in plate tests, it is advisable to distribute the points on the curves randomly and also to include a reference standard in each plate (Gaines-Das and Meager, 1995). One of the most widely used techniques to validate a bioassay's performance is to include internal duplicates. The data arising from the comparison can be important in assessing the test's variability.

Products manufactured by different companies, even with the same source materials, can present very different specific activities. Consequently, the concept of a universal standard of potency has been developed by the World Health Organization (WHO) and this has proved extremely valuable to reduce the variability of results between laboratories, as well as to improve the comparability of clinical studies and research (Mire-Sluis *et al.*, 1996).

The international units and standards are vital when establishing the biological potency of a preparation. The mass cannot be used, since it is practically impossible to weigh a drug, especially if it contains excipients, as occurs with most biotherapeutic products. To establish international standards, a strict process is followed according to the regulations of WHO. The importance of this is reflected in the establishment of new standards, the Reference Reactives (RRs) of the WHO, for cytokines and growth factors. These are prepared according to the WHO protocol. Each standard is monitored so that it does not lose activity during lyophilization, and its activity remains stable. However, the potency of a therapeutic product measured by an *in vitro* test is not used to establish clinical doses, because this may not reflect *in vivo* activity, which can only be established by clinical trials.

13.5.4 Statistical analysis

The most widely used type of simultaneous assay is the one in which the response has a homoscedastic linear regression on a logarithmic scale. Homoscedasticity means that the variance of all experimental groups is the same. For such an assay, the condition of similarity requires that the straight lines of the standard and the samples should be parallel. Otherwise, the condition of similarity between the sample and standard is not established, that is, it would not be valid to assume that dilutions of one behave the same as dilutions of the other, which is the assay's underlying principle.

This type of test is called parallel-line assay and is based on the comparison of a sample response with that of a reference standard (Finney, 1978). In general, it determines the response – at least by duplicates – of a series of dilutions of each preparation (sample and standard) while plotting the means of their corresponding doses on a logarithmic scale. As this test requires analysis of a linear portion of the curves, at least three points of each curve belonging to such a portion should be selected. The more selected points, the better the comparison.

If successive doses for each preparation show the same logarithmic spacing this enables a mathematical transformation that facilitates the calculation without modifying the final result. With these new values assigned to the doses on the abscissa axis and the mean response values on the ordinate axis, the straight lines of dose-response are replotted, and must be parallel to allow comparison. This graph allows a quick potency estimate, and also protects against serious mistakes or misinterpretations of statistical analysis. The horizontal distance between both straight lines provides an estimate of the difference in the transformed abscissa values, between doses with the same response. Finally, by expressing this difference in terms of doses, the sample's potency value is obtained. For this estimate to be valid, the variance analysis – more commonly known as ANOVA – must be performed. The existence of homoscedasticity is determined by Bartlett's test (Finney, 1978).

References

Alwine JC, Kemp DJ, Stark GR (1977), Method for detection of specific RNAs in agarose gels by transfer to diazobenzyloxymethyl paper and hybridization with DNA probes, Proc. Natl Acad. Sci. U S A 74:5350.

Amadeo G, Oggero Eberhardt M, Zenclussen ML, Robles L, Pereira Bacci D, Kratje R, Etcheverrigaray M (2004), A single monoclonal antibody as probe to detect the entire set and partially unfolded rhEPO glycoforms, J. Immunol. Methods 293:191 205.

Bollati Fogolín M, Forno G, Nimtz M, Conradt HS, Etcheverrigaray M, Kratje R (2005), Temperature reduction in cultures of hGM CSF expressing CHO cells: effect on productivity and product quality, Biotechnol. Prog. 21:17 21.

Bradford M (1976), A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding, Anal. Bio chem. 72:248 254.

Cannon JG, Nerad JL, Poutsiaka DD, Dinarello CA (1993), Measuring circulating cytokines, J. Appl. Physiol. 75:1897 1902.

Canosi U, Mascuia M, Gazza L, Serlupi Crescenzi O, Donini S, Antonetti F, Galli G (1996), A highly precise reporter gene bioassay for type I interferon, J. Immunol. Methds 199:69 76.

CBER (Center for Biologics Evaluation and Research) (1993), Points to Consider in the Characterization of Cell Lines Used to Produce Biologicals, US Food and Drug Administration.

Chaplin MF, Kennedy, JF (1994), Carbohydrate Analysis. A Practical Approach, 2nd Ed., IRL Press, Oxford, UK.

Dolnik V (2006), Capillary electrophoresis of proteins (Review 2003 2005), Electro phoresis 27:126 141.

Edman P (1950), Edman degradation, Acta Chem. Scand. 4:283.

Etcheverrigaray M, Amadeo G, Didier C, Pereira Bacci D, Cavatorta FA, Kratje R (2005), Physicochemical estimation of rhEPO potency produced in suspension culture, In: Gòdia F, Fussenegger M (Eds), Animal Cell Technology Meets Genomics, Springer, Dordrecht, pp. 727 729.

Finney DJ (1978), Parallel line assays, In: Statistical Methods in Biological Assays, Charles Griffin, London, pp. 69 104.

Gaines Das RE, Meager A (1995), Evaluation of assay designs for assays using microtitre plates: results of a study of in vitro bioassays and immuno assays for tumor necrosis factor, Biologicals 23:285 297.

Goldfarb R, Saidel L, Mosovich E (1951), The ultraviolet adsorption spectra of proteins, J. Biol Chem. 193:397 404.

International Conference of Harmonization Guidelines, Q5B Quality of Biotechnolo gical Products: Analysis of the Expression Construct in Cells used for Production of R DNA Derived Protein Products (1995), Q5D Derivation and Characteriza tion of Cell Substrates Used for Production of Biotechnological/Biological Products (1997).

Jeffcoate SL, Corbel MJ, Minor PD, Gaines Das R, Schild GC (1993), The control and standardization of biological products, Proc. R. Soc. Edin. 101B:207 226.

Kjeldahl J (1883), Neue Methoden zur Bestimmung des Stickstoffs in organischen Körpern, Fresenius Z. Anal. Chem. 22:336 382.

Kratje R, Lind W, Wagner, R (1994), Evaluation of the proteolytic potential of in vitro cultivated hybridoma and recombinant mammalian cells, J. Biotechnol. 32:107 125.

Laemmli UK (1970), Cleavage of structural proteins during the assembly of the head of bacteriophage T_4, Nature 227:680 685.

Larsen BR, West FG (1981), A method for quantitative aminoacid analysis using precolumn o phthaldialdehyd derivation and high performance liquid chromato graphy, J. Chromatogr. Sci. 19:259 265.

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951), Protein measurement with the folin phenol reagent, J. Biol. Chem. 193:265 275.

Marini G, Forno G, Kratje R, Etcheverrigaray M (2005), Recombinant human granulocyte macrophage colony stimulating factor (rhGM CSF): incidence of glycosylation on pharmacokinetic parameters, In: Proceedings of ENPROMER 2005, Rio de Janeiro, Brazil, pp. 123 124.

Mire Sluis AR, Thorpe R (1998), Laboratory protocols for the quantization of cytokines by bioassay using cytokine responsive cell lines, J. Immunol. Methods 211:199 210.

Mire Sluis AR, Gaines Das R, Gerrard T, Padilla A, Thorpe R (1996), Biological assays: their role in the development and quality control of recombinant biological medicinal products, Biologicals 24:351 361.

Oggero M, Forno G, Kratje R, Etcheverrigaray M (2006), Rational selection of an antibody probe to detect the heterogeneous collection of CHO derived rhGM CSF glycoforms, Biotechnol. Lett. 28:2049 2056, DOI 10.1007/s10529

006 9187 3, electronic online first article.
Pepin R, Lucas D, Lang R, Lee N, Liao M, Testa D (1990), Detection of picogram amounts of nucleic acid by dot blot hybridization, BioTechniques 8:628 632.
Sinclair AM, Elliot S (2004), Glycoengineering: the effect of glycosylation on the properties of therapeutic proteins, J. Pharm. Sci. 94:1626 1635.
Southern EM (1975), Detection of specific sequences among DNA fragments separated by gel electrophoresis, J. Mol. Biol. 98:503 517.
Svensson H (1961), Isoelectric fractionation, analysis, and characterization of ampho lytes in natural pH gradients, the differential equation of solute concentrations at a steady state and its solution for simple cases, Acta Chem. Scand. 15:325 341.
Thorpe R, Wadhwa M, Mire Sluis AR (1997), The use of bioassays for the character ization and control of biological therapeutic products produced by biotechnology, Dev. Biol. Stand. 99:79 88.
Wadhwa M, Bird C, Page L, Mire Sluis AR, Thorpe R (1995), Quantitative biological assays for individual cytokine, In: Balkwill FR (Ed.), Cytokines: A Practical Approach, IRL Press, New York, pp. 357 391.
Whiteside TL (1994), Cytokine measurements and interpretation of cytokine assays in human disease, J. Clin. Immunol. 14:327 339.
WHO (World Health Organization) (1991), Guidelines for assuring the quality of pharmaceutical and biological products prepared by recombinant DNA technol ogy, WHO Technical Report Series, 814:59 71.

Regulatory aspects

14

Maria Teresa Alves Rodrigues and Ana Maria Moro

14.1 Introduction

The scientific and technological achievements of the last decades have allowed the discovery and production of new biological medicines, or substitution of those previously extracted from animal sources, like insulin and growth hormone. Most of those medicines are proteins obtained by animal cell cultivation, which differs from bacteria and yeast in that animal cells carry out post-translational modifications needed for biological activity and similar to the natural protein. Biopharmaceutical production in mammalian cells, including hormones, monoclonal antibodies (mAbs), vaccines, and other molecules with medical interest, involves high cost processes due to factors such as:

- low cell yield
- complex culture media
- shear sensitivity
- low resistance to toxic metabolites
- complex purification
- rigorous control of all phases
- sophisticated methodologies of process control and final product control.

Concepts like quality, safety, and efficacy should be taken into consideration during process development, since it is not enough to inspect and test only the final product. Good Manufacturing Practice (GMP), as well as quality assurance systems are essential to guarantee safety, quality, and economic feasibility during the whole process and therefore to obtain a marketing license.

Biopharmaceuticals, like synthetic pharmaceuticals, can cause adverse effects. Protein molecules can elicit immunological reactions in patients, which can be enhanced by small modifications of the molecule. Thus, it is very important to proceed with in-process controls, final product testing, and process validation. By the use of refined analytical methodologies it is possible to change dogmas such as ''the process defines the product,'' which is imperative for old biological products composed of complex and less purified mixtures. The more recent biopharmaceuticals might be considered as ''well characterized.'' During the last few years considerable discussions have occurred between regulatory agencies and manufacturers concerning the requirements needed for product safety, so regulations are constantly evolving due to advances in research. In this chapter we present the latest positions concerning animal cell-derived biological products

adopted by the regulatory agencies of the major pharmaceutical markets in the world – the FDA (Food and Drug Administration) in the United States and EMEA (European Medicines Evaluation Agency) in the European Union – as well as by the WHO (World Health Organization) and ICH (International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use).

14.2 Good Manufacturing Practices and quality assurance

Quality, efficacy, and safety of a medicine are attributes to be planned and established throughout the production process. This concept is especially valid for the production of biologics to assure the fundamental characteristics of efficacy and safety to the patient in each vial of every lot. Every step of pharmaceutical manufacturing should follow high standards intended for production consistency and product quality. The principles defining these standards are described in the ''Good Manufacturing Practices'' (GMP) publications.

GMP principles were originated in the US, by the FDA, in 1978. The principles are generally accepted throughout the world. Regulatory authorities verify the adherence and compliance to these principles, regularly inspecting the production plants, and determine whether a manufacturing license is maintained or revoked. GMP principles are generic and applicable to all pharmaceutical processes, but may be complemented by specific conditions depending on products or systems. As an example, the European Union publication (EC, 1998a) includes the following chapters: ''Quality management'', ''Personnel,'' ''Premises and equipment,'' ''Documentation,'' ''Production,'' ''Quality control,'' ''Manufacture and analysis contract,'' ''Complaints and product recall,'' and ''Self-inspection.'' In addition to the main chapters, there is a series of annexes: ''Production of sterile products,'' ''Production of biological products for human use,'' ''Production of medicines in development intended for clinical trials,'' ''Production of human blood and plasma derived products,'' ''Computerized systems,'' ''Parametric release,'' among others. Similarly, the FDA publishes the ''Points to Consider'' (PTC) and ''Letters to Industry'', and WHO publishes specific annexes.

The GMP principles are part of a wider concept that encompasses many aspects that can influence product quality; this concept constitutes the ''quality assurance'' system (QA). QA is the sum of systematic actions, which are intended to guarantee quality standards expected for a given product. The QA system concerns all steps that can influence biopharmaceutical quality, such as: research and development; project evaluation under GMP rules; designation of responsibilities with definition of positions and functions; selection of suppliers; raw material storage; process development; validation; pilot scale production; clear description of production and control operations following GMP requirements; testing and control of raw materials, intermediate product, bulk product, and finished product; records; labeling; distribution; and complaints. Self-inspection is also part of the program aiming to evaluate the effectiveness and application of QA system. The QA system should include formal training of produc-

tion and QA personnel, making the whole staff conscious of these practices and their importance for quality attainment. The concepts of QA, GMP, and quality control (QC) are inter-related aspects of quality management.

QC is a segment of the QA system and encompasses sampling, inspection, test and release of raw materials, packaging materials, and intermediate, bulk, and finished products. The final product release depends on aspects like critical examination of production conditions, results of in-process assays, manufacturing documentation, adherence to the specifications, and examination of final packaging. The QC department should be independent of other departments and be granted access to the production areas whenever considered necessary for sampling or investigation, and should be involved in all decisions related to product quality (EC, 1998b; Christiansen, 1999; ANVISA, 2003).

The manufacture of biological products, unlike that of pharmaceuticals, uses materials that present variability. The active substance is generally produced in small quantities and needs to be separated from complex mixtures containing several types of contaminants. Lots are generally small and quality control tests are usually based on biological techniques that present higher variability than physicochemical assays. The processes are also susceptible to microbial contamination. The in-process controls are of fundamental importance to detect quality deviations that cannot be assessed through assays performed only on the final products.

The development of biological products obtained through animal cell culture, such as recombinant proteins and mAbs, has resulted in well-characterized, high purity therapeutic products. The purification processes generally include various chromatography steps, and the products are analyzed by sophisticated analytical techniques and validated by biological activity assays. In 1995, the concept of a ''well-characterized product'' (WCP) was introduced by the FDA, being defined as an entity whose purity, potency, and quantity can be determined and controlled. For a biological compound to be considered a WCP, certain tests and data are not needed, making it simpler to introduce changes to the manufacturing process. To be granted a WCP profile, a product must meet some criteria defined by the industry and the FDA. The manufacturing process should be robust, reproducible, and validated. Validation is used to demonstrate product safety when produced with consistent standards (for example, demonstration of the removal of impurities and adventitious agents through validated analytical tests). Also, the product should be safe from a toxicological perspective and should pass characterization tests to confirm identity, purity, and potency (Foulkes and Traynor, 1999; Schiff, 2004). For recombinant DNA-derived proteins, the identity can be assessed by primary and secondary structure determination, including amino acid sequence analysis, presence of disulfide bonds, and post-translational modifications such as glycosylation. mAbs can be identified by physicochemical and immunochemical assays. Purity and impurities should be quantifiable and the impurities should be identified whenever possible; the quantity and biological activity should be measurable. When a product satisfies these criteria, it is considered ''well characterized'' and can be regulated with less interference from the FDA (Henry, 1996).

14.3 Regulatory agencies

The US regulatory authority for medicines is the FDA. Inside its organizational structure, biological products such as vaccines, blood-derived products, antisera, toxins, and antitoxins intended for therapeutic use are under control of the Center for Biologics Evaluation and Research (CBER). mAbs for *in vivo* utilization, immune modulators, and therapeutic proteins like cytokines, growth factors, and enzymes are generally under the surveillance of the Center for Drug Evaluation and Research (CDER). The FDA regulates clinical trials, evaluates investigational new drug (IND) applications, analyzes clinical data, checks commercial licenses, inspects and approves industrial installations, and verifies GMP adherence.

In the European Union, the regulatory authority is the European Commission (EC), composed of representatives of each participating country. The role of the EC is to propose legislation for the European Union. For pharmaceuticals it has a ten-volume series, named *The Rules Governing Medicinal Products in the European Union* (EC, 2007). These volumes comprise all aspects of pharmaceutical activity. In 1995 the creation of the EMEA (European Medicines Evaluation Agency, later named European Medicines Agency), based in London, simplified the requirements needed for new drug registration. EMEA, like the FDA, receives applications for the marketing of pharmaceutical products. Although they have similar functions, the FDA has the authority to grant a license, while EMEA does not. Through the CPMP (Committee for Proprietary Medicinal Products), the EMEA advises on product licensing, but only the EC has authority to grant a marketing license that covers the European Union, which constitutes the largest pharmaceutical market in the world, followed by the USA and Japan. The PAB (Pharmaceutical Affairs Bureau) regulates the pharmaceutical industry in Japan.

In Brazil, the biological products are regulated by ANVISA (National Agency for Sanitary Vigilance), which has the authority for regulation, control, and inspection of products and services that can be hazardous to public health. It is linked to the Health Ministry, and was created in January 1999. The resolution RDC 315 (ANVISA, 2005) regulates biological products including vaccines, hyperimmune sera, blood-derived products, biomedicines, and allergens. The biomedicines include products obtained from biological fluids or tissues of animal origin, as well as mAbs and others obtained through biotechnological processes. Biological products from other countries can be registered in Brazil if they are registered and licensed in their country of origin. New medicine registration and approval is based on the Medicines Technical Chamber report.

14.4 Harmonization

The goal of the pharmaceutical industry to compete on the international market is hampered by differences among the regulatory requirements. To bring uniformity, the ICH has been promoting a process of harmonization of the technical requirements for pharmaceuticals registration among pharmaceutical industries and regulatory authorities from Europe, the

USA, and Japan since 1990. This effort is supported by the pharmaceutical companies because it will make for more efficient regulation in the most attractive markets and will also be beneficial to patients with faster availability of new drugs. The ICH aims to eliminate duplication of assays during research and development of new drugs, as well as provide guidelines intended to unify the requirements for approval (Walsh, 2004a; Foulkes and Traynor, 1999).

14.5 Premises

14.5.1 Clean rooms

Due to the inherent variability of animal cell-based systems, in-process control and strict adherence to GMP are of critical importance for obtaining quality products. Carefully planned facilities, together with bioreactor design and in-process control systems, determine product quality at feasible manufacturing costs. The regulatory agencies (EC, 1998a; CFR, 2003) clearly state that the premises and equipment must follow GMP standards.

As most biopharmaceuticals are delivered by the parenteral route, production plants must follow rules for ''injection'' quality. Critical operations are done in clean areas. Other facilities, such as storage, packaging, and quality control, should be adequately built and maintained within appropriate conditions of cleaning and hygiene (Walsh, 2004b). Clean areas are defined by the number of particles per unit volume of air. Thus, the main element of a clean area is filtered air. Clean rooms are designed to protect the product from contamination by microorganisms or particles, which may be derived from various sources, air-borne, personnel, or equipment. HEPA (high efficiency particulate air) filters are used to filter the air and remove particulate matter. Sterile products are made in rooms with positive pressure intended to avoid the entry of contaminants from outer rooms. For manipulating pathogens, toxic substances or radioisotopes, the recommendations are to conduct operations in a negative pressure area, with air flow exhaustion through sterilizing filters that should be treated after use (usually incinerated).

Each operation of a production process takes place in a different room within the clean area and might require a specific and defined environment and cleanliness. Rooms should be designed to maintain a degree of cleanliness higher than required when not in use, so they are within limits during operational use. Clean rooms intended for production of sterile products are classified according to the total and live particles in the environment. In Europe (EC, 2003) clean rooms used to be classified as A, B, C, and D; A being for the most critical operations like filling and aseptic connections, while C and D areas were intended for most of the operations, upstream, downstream, and solutions preparation. The corresponding US classification (IES, 1992) was to designate a clean room as class 100 (equivalent to A/B above), class 10.000 (level C) or class 100.000 (level D). Brazilian rules (ANVISA, 2003) used to follow the European and WHO norms (WHO, 2002).

A harmonized classification was issued recently with ISO 14644-1, with reference to the classification terminology and also with units (now in m^3). Rooms classified as ISO class 5, 7, or 8, correspond respectively to US categories 100, 10.000, or 100.000 (SBCC, 2005). The key element of the ISO class 5 is laminar air flow, a mass of air that flows at a controlled and low rate and so drags particles without turbulence. These are generally represented by air flow cabinets (islands of ISO class 5).

Another point to consider when designing a clean room is the material used in construction. Internal surfaces (walls, ceilings, and floors) should be formed by unbroken surfaces made of smooth materials with no shedding particles, therefore allowing easy cleaning and disinfection. Corners should be round. The installations should have a minimum amount of shelving and equipment designed to reduce dust and facilitate cleaning. Recommended materials are polished stainless steel or solid resins.

The flow of materials and people must be carefully managed. Following GMP principles, materials should pass through an airlock that does not allow both doors to be open simultaneously. Used material and products should be removed by an independent exit. Personnel movement occurs through separate rooms with separate doors for entry and exit.

The garments used by the workers should be made of synthetic fabrics that minimize contamination risks. Operators should be trained for their work and also for the maintenance of clean conditions and hygiene. Cleaning is a key factor in the maintenance of good conditions and reduces or eliminates potential contaminants that can affect the product. Contaminants can have various sources: live particles, inert particles (dusts, glass, file dust, lubricants, etc.) or cross-contamination when different processes or products share the same clean area. To avoid problems, the cleaning procedures should be written and approved, validated, and executed by trained operators.

14.5.2 Biosafety

Cell cultivation is a tool for the production of viral vaccines, tumor antigens, mAbs, and recombinant proteins. These organisms, their products, or adventitious agents that may be present in the production processes can potentially be transmitted to the operators, to the environment, and to the outside. Protection measures must be taken to reduce this risk, so that containment must be a central issue regarding the design of facilities, bioreactors, and downstream processing equipment. To work with GMOs (genetically modified organisms) requires adherence to specific rules adopted by the regulatory agency of each country, for example, NIH (2002) in the USA and CTNBio (1997) in Brazil. When working with non-pathogenic recombinant DNA in mammalian cells (risk class I), biosafety level is classified as BSL-1 or BSL-1/''large volume'', when the working volume exceeds 10 L and is in a closed system like a bioreactor. Some procedures, such as sampling, reagent addition, or liquid transfer, are critical and should be performed with particular care to avoid contamination. Exhaust gases from bioreactors should be filtered through HEPA filters or incinerated. GMO-containing bioreactors should only be opened

after adequate sterilization. Waste liquid that could contain live recombinant or pathogenic organisms should be decontaminated before discarding. Simultaneous processing can be accepted for some products like mAbs and recombinant proteins once closed systems are used.

14.6 Cell banks

The production of heterologous proteins for therapeutic use requires selection of the producer cell line, based on yield, monoclonality (for proteins), product quality, stability, and absence of contaminants like bacteria, molds, mycoplasmas, and viruses. Progress in the production of biopharmaceuticals by cell culture is due mainly to the use of diploid cells and continuous cell lines, together with the maintenance of cells by cryopreservation. It is important to guarantee that the expression system chosen is able to generate the product in a consistent and economically feasible way (Levine and Castillo, 1999).

The cells used for a process must be held in a cell bank, guaranteeing a continuous supply during the lifespan of the product. According to the FDA (1993a), the master cell bank (MCB) is defined as a large number of aliquots containing cells with uniform composition and derived from a single tissue or cell and conserved in liquid nitrogen (–196°C). The MCB is used for the preparation of the working cell bank (WCB) that is used for the manufacture of production batches. For the preparation of the banks, cells are cultured to an amount sufficient to dispense into hundreds of individual vials.

The documentation related to the cell banks should include the inventory of all vials, their identity, and their location. There must be a monitoring program for the stability of the cryopreserved cells in the MCB, including viability and growth characteristics. The cell banks must be generated in a controlled environment, observing GMP requirements. While preparing the banks no other cell line or virus should be cultivated simultaneously in the same laboratory or by the same operators. Access to the stored vials should be controlled. The storage temperature should be monitored and the liquid nitrogen level verified in all the storage units, with records of deviations and any correcting actions.

The manufacturer must establish the maximum cultivation span for a particular cell *in vitro* (passage number). The characterization must include the growth rate, morphology, specific yield, and quality of the molecule of interest (Wiebe and May, 1990). The post-production cells (PPCs) must be removed from the bioreactor and tested at least once to evaluate whether new contaminants were introduced or induced by the cultivation conditions. Changes in the culture medium or production scale require new evaluation of the PPCs to determine any effects on the yield and product consistency (Levine and Castillo, 1999).

14.6.1 Cell bank qualification

The cell bank qualification must consider many factors including the presence and identification of contaminants (FDA, 1993a; ICH, 1998a;

WHO, 1998). Usually an extensive characterization is required for the MCB, including tests for the establishment of cell properties and stability of those properties during the production process. The WCB must be subjected to tests such as microbial sterility, including mycoplasma detection tests and routine tests for virus detection (*in vitro* and *in vivo*), in order to identify contaminants that might be introduced by the culture media or manipulation. This is important because the composition of culture media is changing, with the trend to substitute animal sera for chemically defined or animal component-free formulations. For cell line selection for the production of biologicals, FDA (1997) and WHO (1998) do not allow the use of cells obtained from carriers of transmissible diseases or diseases of unknown etiology. Cells from neural origin can contain or are able to amplify the causative agent for spongiform encephalopathy and therefore must not be used for the production of biologicals.

Cell line characterization

The cell line should have a detailed history, including origin (organ, tissue, age, gender, and donor species) and a description of the methods used for its screening, history of passages, and culture media used. The cell characteristics that are identified include: karyotype, isozyme profile, growth rate (generation time), morphology, tumorigenicity, or genetic markers (DNA fingerprinting). When dealing with recombinant products, the documentation should include information about the DNA insert that codes for the product, preparation method, and the complete DNA sequence (vector), including the regulatory sequences and antibiotic resistance markers. The history must contain the cloning details of the recombinant cell and the methodology used, as well as insert copy number and the physical status of the vector inside the host cell, whether integrated or extrachromosomal. The nucleotide sequence of the insert that codifies the expressed product, as well as the restriction map of the expression vector, must be analyzed at the characterization phase of the MCB and at least once in the PPCs (FDA, 1985; ICH, 1995, 1998a; Onions, 1997). Cells such as human epithelial cells used for attenuated vaccine production need to be tested for tumorigenicity (FDA, 1993a). Whenever cells are characterized as non-tumorigenic, *in vivo* and *in vitro* testing are performed either with MCB or WCB cells propagated up to or over the cultivation time allowed for production, according to the methodology described by WHO (1998).

Adventitious agent testing

Cells in culture are susceptible to microbial contamination from various sources. The cell line itself can contain viruses derived from the animal it originates from. Other sources of animal contamination are sera, additives to the culture media, or porcine trypsin used for detachment of adherent cells. Operators represent another contamination source. Bacterial and fungal contamination should be tested in 1% of the total number of ampoules, but not less than in two ampoules of MCB and WCB, according to the protocols described in the European, Japanese, or American

Pharmacopeia (ICH, 1998a). The absence of mycoplasmas must be verified in the MCB and WCB ampoules by at least two different methods (FDA, 1993a). Mycoplasma contaminations are rather common and persistent while not causing perceptible morphological changes. They can cause chromosomal alterations, as well as changes in cellular metabolism and viral replication. According to the literature, the most common sources of contamination are oral human microbiota due to operator contamination, and bovine microbiota originating from sera (Timenetsky *et al*., 1992).

Virus testing

Routine testing for adventitious viruses

Cells derived from MCB or WCB must be tested for adventitious viruses by *in vitro* and *in vivo* methods. For the *in vitro* test, indicator cells can be used to verify cytopathic or morphological changes when challenged with the product, the cells used for production or other related materials, like culture supernatant or lysed cells. The *in vivo* testing for viral agents that induce pathologies can be carried out by the intramuscular inoculation of cells or cell lysates into adult and newborn mice and guinea pigs. An alternative is the intracerebral inoculation of mice with the test material. Any abnormality observed in the clinical state or mortality of the animals indicates the possibility of infection that needs to be investigated. For egg inoculation, viable cells should be injected into the allantoid cavity and yolk sac of embryonated chicken eggs. Cells from MCB and WCB are accepted when neither animals nor eggs show evidence of any viral agent (Onions, 1997; WHO, 1998).

Testing for specific viruses

Species-specific viruses potentially present in mouse, hamster, and rat cell lines should be tested through assays for antibody production in the respective animals (MAP, HAP, or RAP). In this assay the cells are inoculated into an animal of the corresponding species, which is bled after 28 days to evaluate the presence of antibodies against the specific viruses (WHO, 1992, 1998; ICH, 1997). For the lymphocytic choriomeningitis virus, murine cytomegalovirus, murine rotavirus, thymic virus, and lactic dehydrogenase virus, specific tests are recommended (Onions, 1997). The MCB should not be used for production if any potentially human disease-causing virus or polyomavirus is found. Other viruses present do not prevent cells from being used, since removal or inactivation procedures during the downstream processing can be validated. For human cell lines the tests should include Epstein–Barr virus, cytomegalovirus, hepatitis B and C, herpes 6 virus, and any other viruses derived from the donor's anamnesis. Hybrid cells derived from two species should be tested for viruses specific to both species (FDA, 1997; WHO, 1998). Animal-derived reagents like sera or trypsin should be certified through appropriate testing (WHO, 1990). Specific detection tests are required for bovine diarrhea virus, bovine polyomavirus, and prions. Prions are agents of bovine spongiform encephalopathy (BSE) and present a particular risk. It is recommended to use sera exclusively from non-endemic countries of BSE or, even better, not to use animal-derived reagents at all (WHO, 2003).

Today, many media suppliers offer serum-free and/or animal component-free media, thus decreasing contamination risks.

Testing for retroviruses

Retroviruses are associated with oncogenic and degenerative diseases and may be present in some cell lines. They represent a problem since some retroviruses are able to replicate in human cells. Cells from the MCB and cells cultivated over the regular *in vitro* cultivation period (passage number) should be tested for rodent retroviruses by electronic microscopy and infectivity on susceptible cells (transformation focus detection in S+L- cells, as well as syncitium and plaque formation in XC cells). If infectivity is not detected and electronic microscopy does not reveal retroviruses or retrovirus-like particles, a reverse transcriptase assay can be used to detect non-infectious retroviruses (ICH, 1997). Myelomas used for production of mAbs have the inherent capacity of producing infectious retroviruses, and therefore a suitable test should be performed in several supernatant harvests and lot-to-lot consistency should be demonstrated. At the end of a typical cultivation process the burden of endogenous viral particles should be determined. These data are important for planning the purification process, including steps for removal or inactivation of retroviruses (FDA, 1997; WHO, 1998). The CHO cell line expresses defective retroviral particles; although CHO retroviruses are not infectious, recombination is frequent and DNA repair can occur. Thus, for the purification of CHO-derived recombinant proteins, it is recommended to include one or more robust steps for retrovirus removal or inactivation. For cells other than murine, specific assays for retroviruses are necessary. Cells from human origin should be tested for human T-lymphotropic retrovirus (HTLV-1 and -2) and for the lentiviruses HIV-1 and HIV-2 (FDA, 1997; Onions, 1997).

14.7 Validation

The concept of validation came up in the 1970s in association with sterilization procedures and was extended to all steps of pharmaceutical manufacturing procedures. Validation means proving that any and all procedures, processes, equipment, material, operations, and systems comply with the expected performance. Well-planned and well-conducted validation studies constitute GMP principles once they guarantee a consistently safe and efficacious final product. Validation is important for companies, first for QA, and also for cost reduction, decreasing failures, rejection, reworks, recalls, and complaints. The positive aspect of validation is an increase in productivity, as a consequence of a well-controlled process. Validation is required by the regulatory agencies of many countries.

14.7.1 General aspects

A validation plan (Validation Master Plan) is a GMP requirement, attesting control of all critical operation aspects. Consistent results must be

demonstrated in at least three studies with representative production runs showing acceptable and comparable results. Unlike other GMP requirements, validation itself does not improve the process. It confirms that the process is adequately developed and under control. Validation processes require the mutual collaboration of all related sectors, such as development, production, engineering, maintenance, QA, and QC (ANVISA, 2003).

Design Qualification (DQ) is the first validation element of a new facility system or equipment, where adherence to the user's specifications and to GMP rules is demonstrated. Installation Qualification (IQ) follows with the verification of adequacy of the area, installation of equipment pipelines, utilities, instrumentation, and conformity of the material used to the project specifications. At the Operational Qualification (OQ) phase, carried out after installation of all equipment, it is verified whether the system, when in operation, complies with the acceptance criteria defined in the validation plan. Once the OQ phase is successfully finalized it is possible to proceed with the calibration procedures, operation and cleaning, operator training, and preventive maintenance program. After IQ and OQ are concluded, it is time for the Performance Qualification (PQ), with the aim of verifying that what was designed, built, and operated results in a product that meets the expected specifications. Production and QC personnel are specially trained for these assessments. The tests can be done with the product of interest or a placebo, and are related to all operations, from raw material reception to product release (EC, 2001).

Process validation

Process validation is usually performed before commercialization (Prospective Validation). Exceptionally, it may be necessary to validate processes during routine production (Concurrent or Simultaneous Validation). Processes already in use can also be validated (Retrospective Validation). To validate a process, a previous validation of facilities, systems, equipment, and analytical methods (ICH, 1996a), as well as previous training of operators (WHO, 1996; EC, 2001), are required.

Cleaning validation

The efficiency of cleaning procedures should be verified through validated analytical testing with enough sensitivity for residues or detection of contaminants at acceptable levels. It is important that all surfaces potentially in contact with the product be cleaned by previously validated methods. The FDA proposes that manufacturers set limits for active principle residues after cleaning and in the product to be obtained at a subsequent production run (FDA, 1993b). A multipurpose plant should be specially monitored to avoid cross-contamination (Doblhoff-Dier and Bliem, 1999).

Changes in validation

Any change in material, product components, equipment, environment, methodology, or assays that could affect product quality or process reproducibility should be documented. It should be guaranteed that, after the alterations, the process results in a product that meets the specifications previously approved. All changes that might affect product quality or process reproducibility require revalidation and therefore should be subject to permission by the regulatory agencies (WHO, 1996; EC, 2001).

14.7.2 Biological products

The norms for medicinal production are particularly stringent. Biological products are composed of complex molecules, produced by cell lines with a relatively recent history, and difficult to characterize. Tests performed only on the final product do not guarantee consistency of production. The purification procedures should be planned and validated for the removal of potential contaminants from diverse sources: cells, culture media, equipment, and reagents used in the purification or even degradation products derived from the protein itself. There are examples of products with unexpected risks that have caused serious problems such as blood contamination by HIV-1 virus between 1980 and 1985 (Bloom, 1984) or the presence of residual infectious viruses in the poliomyelitis vaccine due to inefficient inactivation (Lubiniecki *et al*., 1990).

Potential risks associated with animal cell-derived products

Among the products derived from animal cells, the potential contaminants are associated with: (i) the cell itself (whole cells, viruses, and other transmissible agents, cellular DNA and growth-promoting proteins); (ii) raw materials (adventitious agents such as bacteria, molds, mycoplasma, viruses, foreign proteins, and endotoxins); (iii) reagents used in the process (antibiotics, resin ligands, solvents, sanitizers, inducers, and nutrients). The production planning must include steps for the removal or inactivation of potential risk factors. It is important to identify the introduction, reduction, or concentration of a given risk factor and its content during the process and in the final product.

The regulatory authorities evaluate safety aspects when the application for registration is submitted. During product development it is possible to discuss further with the authorities with the aim of establishing safety limits (Lubiniecki *et al*., 1990; WHO, 1998).

Viruses and other transmissible agents

Virus identification can start during cell bank characterization and continues until the final product is obtained. It is essential to demonstrate that the viruses are not co-purified with the product (FDA, 1993a, 1997; ICH, 1997). The efficiency of removal or inactivation procedures should be demonstrated on a small scale through spiking experiments, by the use of the viruses themselves, when identified and possible, or with model viruses which mimic any possible variants: large or small, DNA or RNA genome,

enveloped or not, and with variable resistance to physicochemical agents. At least one or preferably more steps for virus removal or inactivation should be included in the purification process. The validation is carried out by deliberate contamination of the supernatant with high titers of the model viruses followed by performing the complete purification protocol. The viral burden is quantified at each step of the process, and the cumulative virus reduction capacity is usually expressed on a logarithmic scale (FDA, 1997).

Cellular DNA

Cells derived from primary cultures or diploid cell lines have been used for a long time for viral vaccine production and the residual cellular DNA is not considered of risk to the users. On the other hand, continuous cell lines, which can be cultivated indefinitely due to deregulation of growth control genes, are thought to pose risks to the users due to the possible transmission of transformation characteristics (Barone *et al*., 1992; Berthold and Walter, 1994). However, Wierenga *et al*. (1995) injected a human tumor DNA into monkeys over an 8-year period without tumor induction. The quantity of DNA administered to a patient undergoing blood transfusion can reach 450 μg (Duxbury *et al*., 1995), which is 10^6 times higher than the limit allowed in products derived from continuous cell lines. These facts, taken together with other evidence (Kurt, 1995; Petricciani and Horaud, 1995; Temin, 1990), led to the argument that the risk associated with residual cellular DNA is low when the amount is 100 pg per dose or less. An important aspect is that residual contaminant DNA usually occurs in small fragments of 200–500 bp (Rodrigues *et al*., 1997), thus not codifying a functional gene.

According to WHO (1998), DNA from a continuous cell line can be considered a cellular contaminant, instead of a significant risk factor that requires reduction to extremely low levels. Therefore, upper limits of 10 ng per dose are acceptable for products generated from continuous cell lines. Only in specific situations that might be considered harmful, for example, when infectious retroviral pro-virion sequences are present, the acceptable limit per dose should be assigned by the regulatory authorities. Levels of cellular DNA should be measured in the supernatant harvest, in the intermediate purification steps and in the final product to determine its initial concentration and whether it was removed or concentrated. The removal efficiency must be determined based on several runs to ensure confidence in the data. Validation of the process excludes the need for residual cellular DNA testing in the bulk product (FDA, 1997; WHO, 1998).

Other contaminants

The presence of transforming proteins that induce the proliferation of different cell lines and that are coded by oncogenes represent a limited risk, as they are secreted in small amounts and are quickly inactivated when administered *in vivo* (Petricciani, 1988; Lupker, 1998). Analytical tests to assess purity as well as the purification process should be validated to demonstrate the capacity to remove host cell proteins to acceptable levels. Once the lot-to-lot consistency has been demonstrated, the test can

be dispensed with for release of the bulk product (WHO, 1998). Other potential contaminants that should be monitored are endotoxins, animal sera-derived proteins or protein fragments, and degradation products or other contaminants derived from the purification process, such as resin ligands, detergents, or salts. The purification steps should be designed to effectively remove those contaminants and the assays for their quantification should be validated (ICH, 1996a).

14.8 Stability

Stability is defined as the period of time during which the product maintains, within the specified limits, the same characteristics and properties displayed when it was manufactured. Environmental factors like temperature, air, light, ionic content, and humidity can affect the drug's quality (Ugwu and Apte, 2004). Proteins are susceptible to proteolysis, denaturation, aggregation, fragmentation, and chemical modifications such as oxidation and changes in the disulfide bonds. The degradation process directly affects the biological activity of the product and might cause immunogenicity, among other adverse effects. The shelf-life is determined by stability tests that guarantee that the product is active, pure, and safe during a specified period, if stored under the prescribed conditions. The stability tests should establish identity, purity, potency, and modifications of the product. Tests to demonstrate stability include: (i) potency of biological activity *in vivo* or *in vitro,* compared to reference material; (ii) purity and molecular characterization of the active component and degradation products, by SDS-PAGE, capillary electrophoresis, immunoelectrophoresis, western blotting, isoelectric focusing, chromatography (reverse phase, ion exchange, hydrophobic interaction, or affinity), peptide mapping, circular dichroism, or mass spectrometry; (iii) physical characteristics and appearance (color, opacity, pH, visible particulates in the solution or after reconstitution of the lyophilized material); (iv) sterility testing or alternatively package integrity testing; (v) assays to quantify additives, stabilizers, preservatives, or excipients.

For registration, the stability tests supporting the proposed shelf-life must be performed in three consecutive product batches in their final package. The data obtained on a pilot scale are accepted for registration with a commitment by the manufacturer, after approval, to submit the first three batches of the commercial-scale production for stability testing. The shelf-life of biological products determines the frequency at which the tests should be repeated. For a product with a shelf-life longer than 1 year, the tests should be repeated every 3 months for the first year, every 6 months for the second year, and annually after that. The stability should be monitored after the beginning of commercialization with enough batches and a frequency to allow a tendency analysis (ICH, 1996b; EC, 2004). Accelerated tests (Tydeman and Kirkwood, 1984), with samples stored at temperatures above the specifications, can help in the identification of degradation products and evaluate the capacity of analytical tools used in the stability testing.

14.9 Clinical trials

The same process that will be used for commercial-scale production should be adopted to produce the material used in the preclinical and clinical trials. Significant differences in the production process used to obtain this material can invalidate the clinical trial results for the commercial product, unless the comparability is demonstrated by identity, purity, stability, and potency tests (ICH, 1998b).

14.9.1 Preclinical studies

Preclinical studies of a new drug give a good indication of its safety before it is used in volunteers. The main objectives of a preclinical trial are to determine the initial safe dose for humans, to define the dose regime, to identify potential toxicity, the organs that may be affected, the severity of adverse effects, and to identify safety parameters for the clinical monitoring. The preclinical studies use two approaches: *in vitro* and *in vivo*. The species specificity, immunogenicity, and pleiotropic activity should be taken into consideration for biopharmaceuticals (ICH, 1998b).

Biological activity/pharmacodynamics

The biological activity can be assessed through *in vitro* assays with cell lines with the objective of determining which effects can be related to the clinical efficacy. The animal species selected for toxicity assays should consider the species specificity of the biopharmaceutical. Mammalian cell lines can be used to anticipate specific *in vivo* activity on other species including the human. For mAbs, immunological properties must be studied, including the antigen specificity, as well as complement binding and cross-reactivity with tissues other than the target. The FDA recommendations (1997) for mAbs list a series of human tissues that must be included in the cross-reactivity testing. A positive result with a non-target human tissue requires an extensive animal study. Preclinical studies for the evaluation of pharmacological and toxicological properties may involve a large number of animals. Consequently, economical and ethical considerations have encouraged the development of alternative *in vitro* systems using cell culture. The guidelines for these studies are being constantly revised by the regulatory authorities.

Toxicity studies

Toxicity studies include the evaluation of acute and chronic effects and reproductive toxicity, as well as mutagenic and carcinogenic activity (CNS, 1997; ICH, 1998b; Walsh, 2004a).

Acute and chronic toxicity

Acute toxicity is determined by the administration of one high dose of the product to rodents, usually rats and mice of both genders. Two delivery routes are chosen, one being that proposed for normal product use. The animals are monitored and any deaths are analyzed in detail.

Studies of chronic toxicity require daily or intermittent administration of the drug, mimicking the clinical use. The doses are chosen within a defined range, ensuring that the higher dose can induce an adverse effect of moderate amplitude and the lower dose does not induce any adverse effect at all. Two species are chosen, usually rats and dogs of both genders, submitted to clinical evaluation with blood and urine analysis. At the end of the study the animals are evaluated by pathological tests. The duration of the study depends on whether the biopharmaceutical is intended for a short or prolonged usage.

Reproductive and teratogenic toxicity

The evaluation of reproductive and teratogenic toxicity, when required, depends on the biopharmaceutical itself and on the end users. Therefore product characteristics should be taken into consideration when designing the study. In cases with potential immunotoxicity for fetal development, the study must include monitoring of newborn immune functions. Generally the reproductive toxicity study verifies the drug effect on the reproductive functionality of males and females. Male spermatogenesis and female follicular maturation are specifically monitored as well as post-mating surveillance to verify possible effects on the fertilization, implantation, and fetal development stages. At least two species are used in the study, generally rodents, mainly rats and rabbits, sacrificed when the gestation period reaches near term, allowing the examination of mother and fetus.

Mutagenic activity

The routine studies for potential mutagenic activity normally performed on chemical drugs do not apply to biopharmaceuticals, as the administration of large amounts of peptides or proteins makes the results difficult to analyze. There is no evidence that these substances interact with DNA or other chromosomal material. In some instances the tests verify the effect of new excipients added to the formulated product.

Carcinogenicity

Conventional tests to verify the carcinogenicity are generally not adequate for biopharmaceuticals. Even so, the tests should be performed depending on the duration of treatment, the target population, and the biological activity of the product, for example, growth factors and immunosuppression agents. The study plan should be adapted to any suspected activity and may depend on specific product characteristics, starting with *in vitro* studies and, if needed, complemented with *in vivo* tests.

14.9.2 Clinical studies

Any new medicine needs to be submitted to clinical trials to determine its safety and efficacy. Clinical research with humans must meet the ethical and scientific criteria defined in ''Good Clinical Practices'' (GCP) (ICH, 1996c). Adherence to the GCP guarantees a trial design of high quality, its implementation, and appropriate divulgence of the results, thus assuring

the rights, safety, and care of the participants. The ICH norm (1996c) contains the requirements of the European Union, Japan, and the USA.

Clinical trials phases

Usually clinical studies follow three consecutive phases, I, II, and III. An additional post-registration study may be required, designated Phase IV (Acceturi *et al*., 1997; CNS, 1997). In some instances, intermediate phases are carried out, I/II, Ia/Ib, etc.

Phase I

The drug is administered to a small group of healthy volunteers with the objective of determining the processes of absorption, distribution, metabolism, and excretion (ADME) and the pharmacokinetic effects. Escalating doses are compared to choose one suitable for both safety and efficacy.

Phase II

This consists of a pilot therapeutic study, following acceptable results in Phase I. This phase aims to evaluate the medicinal effect on patients suffering from the disease or pathological condition for which the biopharmaceutical was developed. It has the objectives of establishing the benefits and efficacy of the drug, the dose-response ratios and the short-term safety. Usually the double-blind technique is used, dividing the patients into two groups, one receiving the biopharmaceutical and the other the placebo, in such a way that neither the physicians nor the patients know to which group they belong. In some instances the placebo is substituted by a previously approved drug for comparison.

Phase III

If Phase II is concluded with safety and efficacy, the therapeutic study is enlarged to include different groups of patients suffering from the disease. Confirmation of safety and demonstration of efficacy are the primary endpoints of Phase III. As it involves a large number of participants, the data analysis should be conclusive on the most frequent adverse reactions, interactions with other medicines, effective doses, advantages over pre-existing drugs, and main factors that affect the results (e.g. age). Phase III is usually an ‘‘open study’’ in which the patients are aware of which medication they are receiving. The competent authorities evaluate the clinical data of the Phase III study to decide whether to grant a license for commercialization or not.

Phase IV

This is a post-marketing study, to establish the long-term safety of a drug, especially when a biopharmaceutical is intended for prolonged use. If unexpected adverse effects are observed the product can be withdrawn from the market. Some Phase III licenses are granted with the condition that an additional study phase should follow according to a predefined plan.

14.10 Biogenerics or biosimilars

A biogeneric or biosimilar medicine is defined as a substance produced after an original patent has expired and with the demonstration of similarity or equivalence to the original product. It is expected to be approved through an abbreviated and simplified regulatory process, requiring lower costs, and to be sold with a generic name at a lower selling price than the branded product. FDA has adopted the name ''follow-on protein product,'' defining it as a product that intends to be a similar version of a protein product already approved.

Patent protection for the first group of biotechnological innovative products has expired or will expire shortly, including interferons, human insulin, erythropoietin, granulocyte-colony stimulating factor, and hepatitis B vaccine. Annual biopharmaceutical sales exceeded 30 billion dollars in 2003 (Dimond, 2003), and this large market has stimulated companies to invest in the development of biogeneric versions of products that have a good market size, a high aggregated cost, and a well-characterized active principle. However, the hurdles for obtaining biogeneric approval are associated with their complexity. Biologics are large proteins, structurally complex, heterogeneous, cell-derived products, attributes that are very dependent on the manufacturing process used to meet the required specifications. Any change in the upstream or downstream steps might lead to protein alterations and the biogeneric manufacturer is posed with the difficult task of proving that the new product is as safe and effective as the original one, that the immunogenicity is not altered, and that there is no detectable difference that could impact safety or efficacy. The requirement for an extension of the clinical and preclinical studies will depend on the nature of the active substance, the formulation, and the molecular complexity, as well as on possible differences from the reference product, including impurities and stability.

The principle that ''the process makes the product'' is mitigated in cases where the product is considered ''well characterized'' and can be easily characterized by well-refined analytical tools. A biogeneric is not identical but comparable. As an example, human growth hormone is being produced by five different companies in Europe through different processes and expression systems; even so, all the processes seem to result in products presenting the same amino acids sequence profile, potency, safety, and efficacy (Polastro and Little, 2001).

The approval of biogenerics for commercialization depends not only on technical and scientific criteria, but also on the balance of political and economic interests. Generic medicines will allow a greater number of patients to benefit from high cost medicines, as biogenerics are expected to be more affordable. Nonetheless the innovative pharmaceutical companies try to postpone the marketing of biogenerics, intending to strategically protect their investments, extending the patents through some modification, reducing prices and innovating by launching a second generation of the original product or a modified formulation intending to present a safer product or a more practical delivery route.

Both the EMEA and FDA are discussing the procedures that should be required for registration of biogenerics. In 2004, EMEA was the pioneer

agency to adopt new rules to authorize the generic version of biopharmaceuticals (Dorey, 2004), directed to the comparability of biological products similar to those approved (EMEA, 2003, 2004, 2005a). The trend is to intensify the post-marketing requirements, especially regarding immunogenicity. A pharmaco-vigilance plan must be considered in the registration application to the European Union for given biopharmaceuticals such as erythropoietin (EMEA, 2005b), granulocyte-colony stimulating factor (EMEA, 2005c), and human growth hormone (EMEA, 2005d). The FDA considers that some biogenerics should follow the same pathway already established for chemically synthesized pharmaceuticals, with the original approval designated as NDA (new drug application) instead of BLA (biologics license application) (Bouchie, 2002a; Evans, 2003). The only consensus is that the biogeneric field is controversial involving many legal considerations. Given this, the FDA has given the opportunity for different interested groups to argue their positions. Workshops (FDA, 2004, 2005a) have been organized to examine the technological and scientific aspects related to the development and marketing of biogenerics. When innovative companies make changes in their processes after the first approval, aiming at optimization, they need to submit comparability protocols (FDA, 2005b) that could be used for the biogenerics companies as well.

Biogenerics of products such as erythropoietin, human growth hormone, hepatitis B vaccine, interferons, insulin, and granulocyte-colony stimulating factor are already available in various countries and regions, such as Korea, India, China, and South America (Bouchie, 2002b; Jayaraman, 2003), nonetheless the entry of these to the American and European market is much more difficult. Omnitrope™, a human growth hormone produced by Sandoz (Novartis group), was the first biosimilar medicine to be approved in Australia in 2004 and in Europe in April 2006 (EMEA, 2006). In May 2006, the FDA set a precedent by approval of the same product, although this followed a court decision and may not suggest easier approval for future biogenerics. The challenge for the biosimilar companies is to ensure the necessary requisites of safety, efficacy, and quality, while allowing access for a greater number of patients to benefit from high cost medicines. To attain these goals, the regulatory agencies must consider concepts like comparability and similarity, which are likely to be dealt with on a case-by-case basis.

References

Acceturi C, Lewi DS, Lousana GB (1997), Manual de Boas Práticas em Ensaios Clínicos, UNIFESP/EPM, São Paulo, Brazil.

ANVISA (2003), Resolução RDC N° 210 de 04 de agosto de 2003. Regulamento técnico das boas práticas para a fabricação de medicamentos, Diário Oficial da União, Brasília, D.F. Available at: http://www.cff.org.br/Legisla%C3%A7%C3%A3o/Resolu%C3%A7%C3%B5es/res 210 2003.pdf (accessed October 2006).

ANVISA (2005), Resolução RDC N° 315 de 26 de outubro de 2005. Regulamento técnico de registro, alteração pós registro e revalidação de registro dos produtos biológicos terminados. Diário Oficial da União, Brasília, D.F., 31 out. 2005.

Available at: http://e legis.anvisa.gov.br/leisref/public/showAct.php?id=19411 &word=Farmacovigil%C3%A2ncia (accessed January 2007).
Barone D, Zaninelli P, Ferrara B (1992), Aspetti regolatori e metodologici della validazione de linee cellulari continue per la produzione de biofarmaci, Minerva Biotec. 4: 82 95.
Berthold W, Walter J (1994), Protein purification: aspects of processes for pharma ceutical products, Biologicals 22:135 150.
Bloom A (1984), Acquired immunodeficiency syndrome and other possible immuno logical disorders in European haemophiliacs, Lancet 1:1452 1455.
Bouchie A (2002a), FDA restructures new review centers, Nat. Biotechnol. 20:960. Available at: http://www.nature.com/nbt/journal/v20/n10/pdf/nbt1002 960a.pdf (accessed October 2006).
Bouchie A (2002b), GeneMedix approval in China, Nat. Biotechnol. 20:213. Available at: http://www.nature.com/nbt/journal/v20/n3/pdf/nbt0302 211.pdf (accessed October 2006).
CFR (2003), Code of Federal Regulations 21: parts 200 299. Available at: http://www. access.gpo.gov/nara/cfr/waisidx 03/21cfrv4 03.html (accessed October 2006).
Christiansen GD (1999), Quality control and quality assurance issues in biopharma ceutical processing, In: Avis KE, Wagner CM, Wu VL (Eds), Biotechnology: Quality Assurance and Validation, vol. 4, Drug Manufacturing Technology Series, Interpharm Press, Buffalo Grove, pp. 33 71.
CNS (1997), Resolução 251/97 de 7 de agosto de 1997. Normas de Pesquisa Envolven do Seres Humanos para a Área Temática de Pesquisa de Novos Fármacos, Medicamentos, Vacinas e Testes Diagnósticos. Diário Oficial da União, Brasília, D.F., 23 set.1997. Seção 1, p. 21.117.
CTNBio (1997), Instrução normativa CTNBio N° 7, de 06.06.97. Normas para o trabalho em contenção com organismos geneticamente modificados OGMs. Diário Oficial da União, Brasília, D.F., 09 jun.1997, Seção I, p. 11.827.
Dimond PF (2003), Manufacturers of Biogenerics Press Forward: But Demand for Bioequivalence Standards May Yet Limit Market, Gen. Eng. News, 23:10 11.
Doblhoff Dier O, Bliem R (1999), Quality control and assurance from the develop ment to the production of biopharmaceuticals, Trends Biotechnol. 17:266 270.
Dorey E (2004), EU rules on biogenerics, Nat. Biotechnol. 22:141. Available at: http:// www.nature.com/nbt/journal/v22/n2/pdf/nbt0204 141.pdf (accessed October 2006).
Duxbury M, Jezuit M, Letwin B, Wright J (1995), DNA in plasma of human blood for transfusion, Biologicals 23:229 231.
EC (1998a), The Rules Governing Medicinal Products in the European Union, Good Manufacturing Practice: Medicinal Products for Human and Veterinary Use, vol. 4, Eudralex. Available at: http://pharmacos.eudra.org/F2/eudralex/vol 4/home. htm (accessed October 2006).
EC (1998b), Quality management, In: The Rules Governing Medicinal Products in the European Union, Good Manufacturing Practice: Medicinal Products for Human and Veterinary Use, vol. 4, Eudralex. Available at: http://pharmacos.eudra.org/ F2/eudralex/vol 4/pdfs en/cap1en.pdf (accessed October 2006).
EC (2001), Guide to Good Manufacturing Practice: Qualification and Validation, Annex 15, Brussels. Available at: http://europs.eu.int/comm/enterprise/ pharmaceuticals/eudralex/vol 4/pdfs en/v4an15.pdf (accessed October 2006).
EC (2003), Guide to Good Manufacturing Practice: Manufacture of Sterile Medicinal Products, Revision to Annex 1, Brussels. Available at: http://europa.eu.int/comm/ enterprise/pharmaceuticals/eudralex/vol 4/pdfs en/revan1vol4 3.pdf (accessed October 2006).
EC (2004), Guide to Good Manufacturing Practice: On going Stability, Addition to Chapter 6, Brussels. Available at: http://europa.eu.int/comm/enterprise/

pharmaceuticals/eudralex/vol 4/pdfs en/2005 10 chapter 6.pdf (accessed Octo ber 2006).
EC (2007), EudraLex The Rules Governing Medicinal Products in the European Union, Available at: http://ec.europa.eu/enterprise/pharmaceuticals/eudralex/index.htm (accessed September 2007).
EMEA (2003), CPMP. Guideline on comparability of medicinal products containing biotechnology derived proteins as active substances: quality issues, London. Available at: http://www.emea.eu.int/pdfs/human/bwp/320700en.pdf (accessed October 2006).
EMEA (2004), CPMP. Guideline on comparability of medicinal products containing biotechnology derived proteins as active substances: non clinical and clinical issues, London. Available at: http://www.emea.eu.int/pdfs/human/ewp/309702 en.pdf (accessed October 2006).
EMEA (2005a), CHMP. Guideline on similar biological medicinal products containing biotechnology derived proteins as active substance: quality issues, London. Avail able at: http://www.emea.eu.int/pdfs/human/biosimilar/4934805en.pdf (accessed October 2006).
EMEA (2005b), CHMP. Guideline on similar biological medicinal products containing biotechnology derived proteins as active substance: non clinical and clinical issues. Guidance on biosimilar medicinal products containing recombinant erythropoietins, Annex, London. Available at: http://www.emea.eu.int/pdfs/human/biosimilar/9452605en.pdf (accessed October 2006).
EMEA (2005c), CHMP. Guideline on similar biological medicinal products containing biotechnology derived proteins as active substance: non clinical and clinical issues. Guidance on biosimilar medicinal products containing recombinant granu locyte colony stimulating factor, Annex, London. Available at: http://www.emea.eu.int/pdfs/human/biosimilar/3132905en.pdf (accessed October 2006).
EMEA (2005d), CHMP. Guideline on similar biological medicinal products containing biotechnology derived proteins as active substance: non clinical and clinical issues. Guidance on similar medicinal products containing somatropin, Annex, London. Available at: http://www.emea.eu.int/pdfs/human/biosimilar/9452805 en.pdf (accessed October 2006).
EMEA (2006), Press release: European Medicines Agency adopts first positive opinion for a similar biological medicinal product. Available at: www.emea.europa.eu/pdfs/human/press/pr/3179706en.pdf (accessed October 2006).
Evans PC (2003), A coming of age for generic biologic formulations? Examining the regulations for expedited approval of generic biological drugs, Gen. Eng. News 23:6,8,17.
FDA (1985), Center for Drugs and Biologics. Points to consider in the production and testing of new drugs and biologicals produced by recombinant DNA technology, Rockville. Available at: http://www.fda.gov/cber/gdlns/ptcdna.pdf (accessed October 2006).
FDA (1993a), CBER. Points to consider in the characterization of cell lines used to produce biologicals. Rockville. Available at: http://www.fda.gov/cber/gdlns/ptccell.pdf (accessed October 2006).
FDA (1993b), Office of Regulatory Affairs. Guide to inspections of validation of cleaning processes, Rockville. Available at: http://www.fda.gov/ora/inspect ref/igs/valid.html (accessed October 2006).
FDA (1997), CBER. Points to consider in the manufacture and testing of monoclonal antibody products for human use, Rockville. Available at: http://www.fda.gov/cber/gdlns/ptc mab.pdf (accessed October 2006).
FDA (2004), CDER Public Workshop: Scientific Considerations Related to Develop ing Follow On Protein Products, September 14 15, 2004. Available at: http://www.fda.gov/cder/regulatory/follow on/default.htm#publicworkshops (accessed

October 2006).
FDA (2005a), CDER Workshop: Scientific Considerations Related to Developing Follow On Protein Products, February 14 16. Available at: http://www.fda.gov/cder/regulatory/follow on/default.htm#publicworkshops (accessed October 2006).
FDA (2005b), CBER Guidance for Industry: Comparability of biotechnological/biological products subject to changes in their manufacturing process, Rockville. Available at: http://www.fda.gov/cder/guidance/6677fnl.htm (accessed October 2006).
Foulkes J, Traynor G (1999), Regulation of antibodies and recombinant proteins, In: Rehm HJ, Reed G, Pühler A, Stadler P (Eds), Biotechnology, 2nd Ed., vol. 5a, Recombinant Proteins, Monoclonal Antibodies and Therapeutic Genes, Wiley VCH, Weinheim, pp. 495 516.
Henry C (1996), FDA, reform, and the well characterized biologic, Anal Chem., Nov 1. Available at: http://pubs.acs.org/hotartcl/ac/96/nov/fda.html (accessed October 2006).
ICH (1995), Steering Committee, Quality of biotechnological products: analysis of the expression construct in cells used for production of rDNA derived protein products, Geneva. Available at: http://www.ich.org/LOB/media/MEDIA426.pdf (accessed October 2006).
ICH (1996a), Expert Working Group (Quality). Guidance for Industry. Validation of Analytical Procedures: Methodology, Geneva. Available at: http://www.ich.org/LOB/media/MEDIA417.pdf (accessed October 2006).
ICH (1996b), Guideline for Industry. Quality of Biotechnological Products: Stability Testing of Biotechnological/Biological Products, Geneva. Available at: http://www.ich.org/LOB/media/MEDIA427.pdf (accessed October 2006).
ICH (1996c), Steering Committee. Guideline for Good Clinical Practice, Geneva. Available at: http://www.ich.org/LOB/media/MEDIA482.pdf (accessed October 2006).
ICH (1997), CPMP. Viral safety evaluation of biotechnology products derived from cell lines of human or animal origin, Geneva. Available at: http://www.ich.org/LOB/media/MEDIA425.pdf (accessed October 2006).
ICH (1998a), CPMP. Quality of biotechnological products: derivation and characterization of cell substrates used for production of biotechnological/biological products, Geneva. Available at: http://www.ich.org/LOB/media/MEDIA429.pdf (accessed October 2006).
ICH (1998b), CPMP. Note for Guidance on Preclinical Safety Evaluation of Biotechnology Derived Pharmaceuticals, Geneva. Available at: http://www.ich.org/LOB/media/MEDIA503.pdf (accessed October 2006).
IES (1992), Institute of Environmental Sciences, Federal Standard 209E, Airborne particulate cleanliness classes in cleanrooms and clean zones, Mount Prospect. Available at: http://www.set3.com/papers/209e.pdf (accessed October 2006).
Jayaraman KS (2003), Indian biogenerics industry emerges, Nat. Biotechnol. 21:1115 1116. Available at: http://www.nature.com/nbt/journal/v21/n10/pdf/nbt882.pdf (accessed October 2006).
Kurt R (1995), Risk potential of the chromosomal insertion of foreign DNA, Ann. N Y Acad. Sci. 772:140 151.
Levine HL, Castillo FJ (1999), Validation of biopharmaceutical processes, In: Avis KE, Wagner CM, Wu VL (Eds), Biotechnology: Quality Assurance and Validation, Interpharm Press, Buffalo Grove, p. 115.
Lubiniecki AS, Wiebe ME, Builder SE (1990), Process validation for cell culture derived pharmaceutical proteins, In: Lubiniecki AS (Ed.) Large Scale Mammalian Cell Culture Technology, vol. 10, Bioprocess Technology, Marcel Dekker, New York, pp. 515 541.

Lupker JH (1998), Residual host cell protein from continuous cell lines: effect on the safety of protein pharmaceuticals, In: Brown F, Griffths E, Horaud F, Petricciani JC (Eds) Safety of Biological Products Prepared from Mammalian Cell Culture, vol. 93, Dev. Biol. Stand., Karger, Basel, pp. 61 64.
NIH (2002), Guidelines for research involving recombinant DNA molecules. Available at: http://www4.od.nih.gov/oba/rac/guidelines 02/nih guidelines apr 02.htm (accessed October 2006).
Novartis (2004), March to global leadership: follow on biologics. Available at: http://www.novartis.com/annual reports/2004/march.shtml (accessed October 2006).
Onions D (1997), Safety evaluation of products derived from mammalian cell lines, In: Hauser H, Wagner R (Eds), Mammalian Cell Biotechnology in Protein Production, Walter de Gruyter, Berlin, pp. 171 190.
Petricciani JC (1988), Changing attitudes and actions governing the use of continuous cell lines for the production of biologicals, In: Spier RE, Griffiths JB (Eds), Animal Cell Biotechnology, vol. 3, Academic Press, London, pp. 14 23.
Petricciani JC, Horaud FN (1995), DNA, dragons and sanity, Biologicals 23:233 238.
Polastro E, Little A (2001), When will we see legal generics of top biopharmaceuticals? ContractPharma Out. Available at: http://www.contractpharma.com/articles/2001/10/the future of biogenerics.php (accessed October 2006).
Rodrigues MTA, Raw I, Moro AM (1997), Residual DNA from hybridoma cultures: decorrence of apoptosis?, In: Carrondo MJT, Griffiths JB, Moreira JL (Eds), Animal Cell Technology: From Vaccines to Genetic Medicine. Kluwer Academic, Dordrecht, pp. 467 472.
SBCC (2005), Classificação de Salas Limpas. NBR ISO 14644 1 cancela e substitui a NBR 13700, Revista da SBCC, 19:16.
Schiff LJ (2004), Biotechnology products derived from mammalian cell lines: impact of manufacturing changes, Regulatory Affair Focus Oct:29 31.
Temin HM (1990), Overview of biological effects of addition of DNA molecules to cells, J. Med. Virol. 31:13 17.
Timenetsky J, Miyaki C, Mendes IF, Rizzo E (1992), Identificação de micoplasmas pela inibição de crescimento de amostras isoladas de culturas celulares, Rev. Saúde Públ. 26:17 20.
Tydeman MS, Kirkwood TBL (1984), Design and analysis of accelerated degradation tests for the stability of biological standards I. Properties of maximum likelihood estimators, J. Biol. Stand. 12:195 206.
Ugwu SO, Apte SP (2004), Efeito de tampões na estabilidade conformacional de proteínas, Pharm. Technol. Brazil 8:56 78.
Walsh G (2004a), The drug development process, In: Biopharmaceuticals: Biochemistry and Biotechnology, 2nd Ed., John Wiley & Sons, West Sussex., pp. 69 91.
Walsh G (2004b), The drug manufacturing process, In: Biopharmaceuticals: Biochemistry and Biotechnology, 2nd Ed., John Wiley & Sons, West Sussex, pp. 97 102.
WHO (1990), Expert Committee on Biological Standardization, Requirements for poliomyelitis vaccine (oral), Tests for bovine viruses in serum, Geneva, Appendix 1: p. 66 [Technical Report Series no. 800].
WHO (1992), Expert Committee on Biological Standardization. Guidelines for assuring the quality of monoclonal antibodies for use in humans, Geneva, pp. 47 67 [Technical Report Series no. 822].
WHO (1996), Good manufacturing practices: guidelines on the validation of manufacturing processes, Geneva, pp. 81 95 [Technical Report Series no. 863].
WHO (1998), Expert Committee on Biological Standardization. Requirements for the use of animal cells as *in vitro* substrates for the production of biologicals, Geneva, pp. 19 56 [Technical Report Series no. 878].
WHO (2002), Good manufacturing practices for sterile pharmaceutical products, Geneva, pp. 76 96 [Technical Report Series, no. 902].

WHO (2003), Working Group on International Reference Materials for Diagnosis and Study of TSEs. Guidelines on Transmissible Spongiform Encephalopathies in Relation to Biological and Pharmaceutical Products, Geneva. Available at: http://www.who.int/bloodproducts/publications/en/WHO TSE 2003.pdf (accessed October 2006).

Wiebe ME, May LH (1990), Cell banking, In: Lubiniecki AS (Ed.), Large scale Mammalian Cell Culture Technology, vol. 10, Bioprocess Technology, Marcel Dekker, New York, pp. 147 160.

Wierenga DE, Cogan J, Petricciani JC (1995), Administration of tumor cell chromatin to immunosuppressed and non immunosuppressed non human primates, Biologicals 23:221 224.

Regulatory agencies web sites

ANVISA:	http://www.anvisa.gov.br
EC:	http://ec.europa.eu/enterprise/pharmaceuticals/eudralex/homev4.htm
EMEA:	http://www.emea.eu.int/
FDA:	http://www.fda.gov/cber/guidelines.htm and http://www.fda.gov/cder/biologics/default.htm
ICH:	http://www.ich.org
WHO:	http://www.who.int/biologicals/en/

Intellectual property

15

Ana Cristina Almeida Müller and Leila Costa Duarte Longa

15.1 Introduction

Intellectual property covers industrial property including author's rights over written material, novel processes, products, computer programs, and integrated circuits. One of the main goals of intellectual property is to promote progress of technology and industry, by granting a temporary monopoly for innovation through patents.

Industrial property is protected through patents covering inventions, utility models, registration of industrial designs, and trademarks. These are protected through rights of exclusivity. Another aspect of protection is through the suppression of unfair competition, which is directed against acts of industrial or commercial competitors contrary to honest practices, including:

(i) acts that can create confusion with a competitor's establishment, products, industrial or commercial activities;
(ii) false claims that can discredit a competitor's establishment, products, industrial or commercial activities; and
(iii) indications or claims that can mislead the public as to the nature of the manufacturing process or the characteristics, suitability or quantity of products.

This chapter discusses the protection of inventions in the field of biotechnology, including processes based on cell cultures. The ethical and moral aspects of patent protection of the results of research involving the use of genetic engineering and the difference between discovery and invention are also discussed. The requirements for patentability are covered, and examples of inventions involving antigens and antibodies are presented. Finally, the importance of internal intellectual property and technology transfer offices and of specialists in these areas is discussed.

15.2 The biotechnology sector

According to the definition of Bull *et al*. (1982), biotechnology can be defined as "the application of the principles of science and engineering for the development of materials, through the use of biological agents, aiming to produce services and goods." Biotechnology is a recent industrial sector, based on the knowledge acquired through research in the areas of biochemistry and biology, particularly molecular biology. It involves some small start-up firms that have a close relationship with university scientists. Even more important, these biotech firms play a key role in

transferring the knowledge generated by university laboratories to the market, through the development of products and/or processes. According to the result of the sixth annual licensing survey carried out by the Association of University Technology Managers (AUTM, 1999) of the United States, sales of products arising from inventions generated by academic research and licensed to industry reached roughly US$ 20.6 billion in 1996 in that country. Additionally, nearly two-thirds of these licenses went to small companies, including biotech firms. This shows how the biotech sector is dependent on partnerships with the academic sector (Müller, 2003).

Biotechnology firms tend to cluster around prestigious universities, particularly in the United States, where Boston (e.g. Harvard and MIT) and the San Francisco Bay Area (e.g. UC Berkeley, Stanford, and UC San Francisco) are noted centers of biotech activity.

For the biotechnology industry, patent rights are important not only because of the monopoly they guarantee, and hence the return on R&D investments, but also in many cases they are the only "product" the company possesses. Patents, then, are of vital importance to obtain financing, whether through venture capital or technology transfer agreements. Additionally, investors look not only at the number of patents, but also the exact knowledge that is protected and the potential value of the subject matter of the patents (Müller, 2003).

Therefore, it is fundamental for companies to have precise targets to decide in which areas to pursue patents and in which areas to invest. Nevertheless, in the case of new start-ups and research institutions, this decision on what to patent is not always an easy task. Taking the shotgun approach of seeking patents on all results can be a waste of time and money. Establishment of a suitable policy in this respect is needed to obtain a good financial return.

15.3 Ethical and moral aspects of research involving genetic engineering

Biotechnology as it is known today traces its roots to the revolutionary research of Cohen (Stanford University) and Boyer (University of California-San Francisco), which resulted in the basic technology of recombinant DNA. This was the start of molecular biology and it has become the basis for genetic engineering.

Genetic engineering is part of biotechnology and can be considered one of the great revolutions of the twentieth and twenty-first centuries. Although the exact definition of genetic engineering is a subject of debate in scientific circles, according to Beier and Straus (1986), it can be considered as the isolation of genetic material with the aim of building new recombinant constructs and to insert these constructs of nucleic acids into live organisms to reproduce them in a new environment.

Genetic engineering takes in a multiplicity of new processes, among them:

(i) processes to obtain recombinant DNA *in vitro*, which basically occurs in four steps: isolating the gene of interest; inserting it into a

vector to obtain a gene transport vehicle, known as a recombinant vector; separation of the successfully ''constructed'' cells from those not transformed, called clone selection; and finally the proliferation of these cells, normally through a culture process to synthesize the product of interest;

(ii) processes to produce monoclonal antibodies (mAbs) with the use of hybridomas; through fusion of lymphocytes that produce antibodies with myeloma tumor cells capable of propagating rapidly;

(iii) hybridization processes using somatic cells, which are based on cell culture experiments and use isolation and fusion of protoplasts, exploiting the totipotency of plant cells; and

(iv) micro-injection processes, in which DNA sequences that have been cloned *in vitro* through recombinant DNA technology are injected into fertilized animal egg cells.

Examples of the products that can be obtained through the processes include: DNA vectors, such as plasmids, viruses, bacteriophages and cosmids; synthetic genes; transformed human viruses (e.g. the Epstein–Barr virus); transformed bacterial cells containing specific properties; animal and plant cells and bacteria; mAbs, tissue cells; regulatory proteins such as human insulin, interferons and human growth hormones; and transgenic plants and animals.

Public perception of this technology includes admiration and anxiety, hope and fear and this can interfere with progress. Nevertheless, despite all the expectations created by genetic engineering, which range from cures for various diseases to the fight against hunger, the public debate in recent years has been dominated by its acceptability and questions on who is responsible for imposing limits. Is it scientists, guided by their own conviction, or should politicians and law-makers intervene? In reality, the entire discussion should be centered on how genetic engineering can promote scientific and technological development in the search for benefits to society. The debate is perfectly understandable because it deals with a new field, one that like any new technology has been the subject of misunderstandings and misapprehensions, and still needs some provisional limits.

In the area of law, economic questions are dominant and the debate on genetic engineering centers on the balance of benefits between interest groups. However, safety and minimization of risks are important, as well as ethical and moral criteria.

In the attempt to promote technical, economic, and scientific development, society uses a special legal instrument, based on patent protection of inventions. Innovation is recognized by granting exclusive rights for a limited period. The inventor is rewarded for having achieved results that are useful to society. Industry, in turn, is encouraged to conduct research. The patent system is aimed to encourage invention, investment, and innovation. This promotes knowledge by revealing and disseminating inventions, which are protected for a fixed period of time from unfair competition.

The central goal is to promote technology, not to regulate it. However, the patent system does not promote technological progress and grant

temporary monopolies to all inventions just because they meet the basic requirements for patentability (novelty, inventive step, and industrial application). Despite being essentially an instrument of economic and technical policy, the patent system is subject to the barriers inherent in the legal system, imposed by constitutions, public policies, and moral perceptions. These barriers are set forth in the legislations of virtually all countries, Brazil included. According to Article 18, I, of the Brazilian Industrial Property Law (Law 9.279/96), the following are not patentable: *"anything contrary to public morality, decency, safety, order and health".*

Nevertheless, it should be clarified that innovations are subject to the general rule that the legal and moral effects of an invention must always be judged in relation to the use normally expected of the patented product or process, not just the possibility of misuse. For example, a specific product that can be legally used as a pesticide is not excluded from patentability simply because it can also be used in chemical warfare.

The Enlarged Board of Appeal of the European Patent Office made the following comments when questioned about a recent case involving transgenic plants (Novartis G 0001/98), in relation to Article 53(a) of the European Patent Convention (EPC), which establishes that inventions that are contrary to public morality and customs are not patentable:

> *"Suppose that a claimed invention defined a copying machine with features resulting in an improved precision of reproduction and suppose further that an embodiment of this apparatus could comprise further features (not claimed but apparent to the skilled person) the only purpose of which would be that it should also allow reproduction of security strips in banknotes strikingly similar to those in genuine banknotes. In such a case, the claimed apparatus would cover an embodiment for producing counterfeit money which could be considered to fall under Article 53(a) EPC. There is, however, no reason to consider the copying machine as claimed to be excluded since its improved properties could be used for many acceptable purposes".*

The same principle applies to inventions related to genetic engineering. Thus, the transfer of exogenous genes by micro-injection, a process frequently employed in genetic engineering, is not excluded from protection just because it can be used to transfer genes to human germ cells (Beier and Straus, 1986).

15.4 Basic concepts of patentability

15.4.1 Discovery versus invention

Discoveries and inventions are increasingly the result of research. But while discoveries are not protected by industrial property or in any other form, and can be known and used freely by all, inventions (and so-called utility models) are protected by patents and their use is restricted: free for use in research and development, but not for industrial production with-

out the owner's authorization. This is a universally accepted concept, and is in general stated explicitly in national laws.

A discovery is the revelation of something that exists in nature, does not aim at pre-established practical purposes, and only increases the sum of human knowledge of the physical world. In contrast, an invention is a solution to a technical problem that seeks to satisfy predetermined ends and practical needs. It can be said that discovery is not the inventive spirit that acts, but the speculative spirit and the faculties of observation, so that with a discovery we are in the field of science and speculative intellect, while with invention we penetrate the domain of engineering and practical intellect (Gama Cerqueira, 1982).

However, American patent legislation differs from others, since it explicitly provides for the possibility of protecting discoveries:

> *35U.S.C. 101 – Inventions Patentable*
> *Whoever invents or discovers any new and useful improvement thereof, may obtain a patent therefore subject to the conditions and requirements of this title.*

15.4.2 Requirements for the patentability of inventions

The basic requirements of national laws on protection of inventions by patents are novelty, inventive step, and industrial application, which are now examined in turn.

Novelty

An invention possesses novelty when the technical knowledge for which the patent protection is sought is not included in the state of the art. Not in the state of the art is usually defined as all the information not available to the public, in any form of disclosure – oral, written, digitized, use/exploitation, among others – at the time of filing the patent application. Absolute novelty is adopted by most countries, meaning that which has not been disclosed anywhere in the world.

Some countries have legal provisions accepting the disclosure of the innovation before seeking protection, as long as the patent application is filed within a reasonable period that is predefined in law. This ranges from 6 months (e.g. Japan) to 1 year (e.g. Brazil and the USA). This term is called the ''grace period.''

In Brazil, the safeguard of the rights of inventors, who are often obliged in some form to disclose their inventions or creations before filing a patent application, is established in the Industrial Property Law, Article 12, I to III (inventions and utility models), and Article 96, § 3 (registration of industrial designs). The provisions of Article 12 are as follows:

> *Article 12 – The disclosure of an invention or utility model occurring during the 12 (twelve) months preceding the filing date, or priority date, of the application shall not be considered to be comprised in the state of the art if made:*

I – by the inventor;

II – by the National Institute of Industrial Property [Instituto Nacional de Propriedade Industrial – INPI], through official publication of an application filed without the consent of the inventor, based on information obtained from the inventor or as a result of his acts; or

III – by third parties on the basis of information obtained directly or indirectly from the inventor or as a result of his acts.

Sole Paragraph – The INPI may require from the inventor a statement relating to the disclosure, accompanied under the conditions established in the regulations.

In spite of the positive aspect conferred by the grace period, it must be noted that not all countries accept this provision or similar procedures. Therefore, even though this possibility exists in Brazil, the filing in other countries that do not accept previous disclosure (the majority of European countries, for example) can be prejudiced. Hence, even in Brazil disclosure before filing a patent application should be avoided.

An interesting example of a case concerned with novelty can be cited from Decision T 301/87 (Interferon α/Biogen) by the Opposition Division of the European Patent Office, in a case involving a patent granted to Biogen (EP 0321 134), where nine opponents requested its cancellation for lack of novelty (OJ EPO. 1990, 335).

The two main claims are as follows:

(1) A recombinant DNA molecule for use in cloning a DNA sequence in bacteria, yeasts and animals cells, said recombinant DNA molecule comprising a DNA sequence selected from:
   (a) The DNA inserts of :
   Z-pBR322(Pst)/HClF-4c;
   Z-pBR322(Pst)/HClF-2h;
   Z-pBR322(Pst)/HClF-SN_{35};
   Z-pBR322(Pst)/HClF- SN_{42} e;
   Z-Pkt287(Pst)/HClF- 2h- AH6, said DNA inserts being exemplified, but not limited to the DNA inserts of the recombinant DNA molecules carried by the microorganisms identified by accession numbers DSM 1699-1703, respectively;
   (b) DNA sequences which hybridize to any of the foregoing DNA inserts and which code for a polypeptide of the IFN-alpha type, and
   (c) DNA sequences which are degenerate as a result of the genetic code to the DNA sequences and inserts defined in (a) and (b) and which code for a polypeptide of the IFN-alpha type.

(2) A recombinant DNA molecule according to claim 1, wherein said DNA sequence which hybridizes to said DNA insert (a) is selected from:
   (d) the DNA inserts of Z-pBR322(Pst)/HcIF-II-206 and Z-pBR322(Pst)/HcIF-SN35-AHL6, said DNA inserts being

exemplified, but not limited to, the DNA inserts of the recombinant DNA molecules carried by the microorganisms identified by accession numbers ATCC 31633–31634, respectively,

(e) DNA sequences which hybridize to any of the foregoing DNA inserts and which code for a polypeptide of the IFN-alpha type, and

(f) DNA sequences which are degenerate as a result of the genetic code to the DNA sequences and inserts defined in (d) and (e) and which code for a polypeptide of the IFN-alpha type.

According to the opponents, the main argument against granting a patent was the lack of novelty of the recombinant DNA sequences claimed, which included the DNA sequences that coded interferon-alpha (IFN-alpha) as an insert. This prior art referred to DNA genome libraries, which are fractioned segments (15–20 Kb) of chromosomal DNA of human fetuses, cloned as derivatives of recombinant λ phages.

However, the opinion of the Technical Board of Appeal was that the prior art cited by the opponents did not compromise the invention's novelty, observing that:

> *"... the mere existence of a DNA sequence coding for a polypeptide of the IFN-alpha type, within the multitude of clones of "Lawn's gene bank" cannot automatically mean that the chemical compound (polynucleotide) concerned does become part of the state of the art. The latter would only then be the case if the existence of the compound concerned had recognizably been made publicly available".*

Hence, according to the Technical Board of Appeal, DNA or genome libraries of an organism do not anticipate the novelty of the DNA sequences contained in and isolated there from.

Inventive step

To meet this requirement, an invention may not be obvious in the sense that the idea would not have occurred to a specialist in the field to which the creation pertains if that specialist were called on to find a solution to the problem resolved by the creation. In other words, the matter to be protected cannot be a simple substitution of matters or means known by others who have conceived the same function, nor a mere combination of known means without a new and unexpected technical effect.

As an example, suppose that it is known to experts in the field that an extract from the plant *Clusia criuva* has anti-inflammatory activity. Therefore, a specialist in the subject would be led to test other species of the genus *Clusia* for the same biological activity (anti-inflammatory). Nevertheless, a way to prove inventive step would be to demonstrate that the species of interest, for example *Clusia rosea*, presents an unexpected technical effect. In other words, while the species *Clusia criuva* has an undesirable side effect associated with its anti-inflammatory action, such

as harm to the intestinal mucous, *Clusia rosea* does not have this disadvantage, and thus can meet the requirement of inventive step.

The stance of the European Patent Office in general is to consider as indicative of the presence of the inventive step the existence of surprising or superior properties, which could not be anticipated by persons skilled in the art. In the case of a new protein, for instance, this might be fewer or less severe side effects, better absorption, or greater stability.

The same reasoning can be applied to the inventive activity involving antigens and antibodies. If the antigen is new and has an inventive step because of surprising properties, then it is patentable. The same goes for a mAb directed against a patentable antigen, since it is new and a person skilled in the art would not have found any suggestion of obtaining it. If on the other hand the antigen (or antiserum) is known, a person skilled in the art would be capable of preparing mAbs against the antigen, without difficulty, based on his or her technical knowledge. A particular mAb will be new in this case, because it has not been described in the state of the art, but will lack an inventive step to the extent that an average specialist in the field could obtain it based on knowledge of the antigen.

However, even if an antigen is known, a specific mAb could still show an inventive step as long as it presents a superior property when compared to other antibodies directed against the same antigen. This superior activity can be evidenced, for example, by the fact that the mAb has a complementary structure to an essential epitope (antigenic determinant) of the antigen's surface, and thus exhibits exceptional binding properties to the antigen. The use of this specific mAb might offer surprising advantages in diagnosing and/or treating a disease caused by the antigen. The selection of this specific mAb, starting from a large number of antibodies aimed against the same antigen, but possessing diverse epitopes, justifies the existence of inventive activity.

The claim for a specific mAb, however, will be inevitably restricted. The surprising technical effect required to establish the presence of an inventive step imposes limits on the possibilities of generalizing the invention, that is, the scope of protection.

It should be pointed out that in cases of mAbs, the complex structures of the corresponding genes, which lead to a vast number and variety of antibodies, generally mean it is impossible to isolate a single mAb a second time, so as to satisfy the capacity for repetition. A complete revelation of the invention to permit it to be repeated – as in the case of a new organism isolated from nature – can only be guaranteed by depositing the hybridoma producing the specific mAb with an internationally recognized depositary authority (IDA) before filing the patent application.

Tables 15.1 and *15.2* summarize the requirements for novelty and inventive step for patentability of DNA sequences and mAbs.

Industrial application

An invention must have a use in economic or industrial production. The term ''industrial'' is used in the broad sense to cover both products and processes, including any economic activity that involves obtaining products in varied sectors besides industry *per se*, such as agriculture, fishing,

Table 15.1 Patentability of DNA sequences that codify a specific protein

Protein	Protection of any DNA sequence		Protection of a specific DNA sequence	
	Novelty	Inventive step	Novelty	Inventive step
New	+	+	+	+
Known			+	+

Table 15.2 Patentability of monoclonal antibodies (mAbs) corresponding to an antigen

Antigen	Protection of any monoclonal antibody		Protection of a specific monoclonal antibody	
	Novelty	Inventive step	Novelty	Inventive step
New	+	+	+	+
Known			+	+
Deposit of the hybridoma	Deposit in an IDA not necessary		Deposit in an IDA necessary	

IDA, international depositary authority.

and mineral extraction, as well as any equipment, instruments or apparatus used in these endeavors.

In addition to the above three requirements, inventors must also clearly and completely describe the invention. This is called sufficiency of disclosure. The specification of a patent application must clearly and sufficiently describe the subject matter, so that it can be carried out by a skilled person in the subject. Some countries (such as Brazil and the United States) also require an indication, when applicable, of the best way of executing the invention, called the best mode of the invention.

Europe does not have the best-mode requirement in the European Patent Convention, meaning the applicant is not obliged to deposit biological material merely with the intent of allowing reproduction of the invention. Rule 28(1) of that convention cannot be interpreted as obliging the deposit of biological material to facilitate reproducing the invention, as long as it can be reproduced from the description (even if this route is more laborious and lengthy than simply growing the deposited material). Such an interpretation would be to introduce the best-mode requirement into European legislation (T 223/92 and T 412/93). It should be pointed out, however, that in opting not to deposit the biological material, the applicant should be sure to provide complete information on the material. There are cases where the European Patent Office's Technical Board of Appeal, after carefully examining an invention's description, has indicated that the information supplied in the application was sufficient to permit a person skilled in the art to reproduce the invention, even in the absence of a deposit of the biological material (T 283/86 and T 181/87). In other cases,

however, the Tribunal has considered the description insufficient (T 815/90 and T 816/90).

In the case of inventions involving new biological materials that cannot be described so as to permit repetition of the invention by a skilled person, it is necessary for the applicant to deposit this material with a depositary institution indicated in an international convention.

The requirement of descriptive sufficiency is covered by Article 24 of Brazil's Industrial Property Law, which reads:

> *Article 24. The description shall clearly and sufficiently describe the subject matter, thus enabling it to be carried out by a person skilled in the art, and shall where appropriate indicate the best manner of execution.*
>
> *Sole Paragraph – In the case of biological material essential for carrying out the subject matter of the application, which cannot be described in accordance with this article and which is not accessible to the public, the description shall be supplemented by the deposit of the material with an institution authorized by the INPI or referred to in an international agreement.*

15.5 Patentable materials

Under the terms of Article 8 of the Brazilian Industrial Property Law, patentable inventions are those that have the three basic requirements of novelty, inventive step, and industrial application. Additionally, from Article 50, II, if the specification does not clearly and sufficiently describe the subject matter so as to enable its reproduction by a technician in the subject, the so-called sufficiency of disclosure, it is against the provisions of Article 24, and is thus not accepted (Art. 50, II).

Article 10 of that law establishes what may not be considered an invention or utility model, and for this reason is expressly excluded from patent protection. According to article 10 (IX) the following is not considered as an invention or utility model:

> *"all or part of natural living beings and biological materials found in nature, or isolated there from, including the genome or germ plasma of any natural living being and the natural biological processes."*

Therefore, Brazil does not yet grant patents to extracts and active ingredients from plants. It does grant use patents, that is, in the form of a pharmaceutical composition, for example, that contain the extract or active ingredient(s). However, this practice of granting patents for extracts and molecules taken from plants is common internationally.

It should be stressed, then, that since patents in this area are being issued in other countries, such as the United States and various European countries, it is advisable to evaluate the territory suitable for seeking protection.

Table 15.3 presents a list with examples of materials considered patentable and non-patentable under Article 10, IX, and Article 18, III, of the

Table 15.3 Patentable and non-patentable material according to Article 10, IX, and Article 18, III, of the Brazilian Industrial Property Law

Patentable inventions	Non patentable inventions
Processes related to transformation of plants	Plant and animal cells
Recombinant genes	Seeds
Transgenic microorganisms*	Human insulin isolated or purified from human pancreas beta cells
Expression vectors that carry genes that codify human insulin isolated or purified from human pancreas beta cells	Genes that code for human insulin isolated or purified from human pancreas beta cells
Escherichia coli transformed by such expression vectors	Yeasts isolated or purified artificially from nature
Processes for production of human insulin involving the culturing of those transformed *E. coli* bacteria	Microorganisms isolated or purified artificially from nature and that produce an antibiotic X
Recombinant proteins	Antibiotic X produced by such microorganism
Mutants obtained by genetic modification of such recombinant human insulin	Human hepatocyte Y
Hybridomas that produce antibodies able to recognize antigen A	Human liver tissue Z
Pharmaceutical compounds containing extracts isolated from plant Y for treating disease Z	Extracts isolated from plant Y

*For the purposes of Law 9279, transgenic microorganisms are those (except all or part of plants or animals) that express, through direct human intervention in their genetic composition, a characteristic normally not attainable by the species in natural conditions.

Brazilian Law 9279/96. According to the latter provision, all or part of living things are not patentable, except transgenic microorganisms that meet the three patentability requirements (novelty, inventive step, and industrial application) set forth in Article 8 of the law and that are not mere discoveries.

Scientific advances have permitted the industrial employment of genetically modified microorganisms or cells, capable of producing proteins of interest in various areas, particularly human health. This technology permits reproducing proteins identical to their natural counterparts, as well as to elaborate others that are totally new by alterations resulting from the insertion of genes into these microorganisms or cells. These genetically modified molecules can be more effective than the natural ones for a predetermined function, for instance through greater biological activity, longer average lifetimes or fewer or less serious side effects.

Before the advent of the current Law 9.279/96, pharmaceutical products were not eligible for patent protection in Brazil (under the former Industrial Property Code, Law 5.772/71). With the new possibility of patenting pharmaceuticals, the huge advance in biotechnology and genetics in recent decades has led to the development of a new and important segment in the chemical-pharmaceutical industry: biopharmaceuticals. Libraries of new compounds from animals, plants, fungi, bacteria, and viruses, which provide additional alternatives to chemical processes and creation of a multiplicity of novel molecules, can be seen.

These advances have brought ethical, economic, and legal implications to the granting of patents for all or parts of living things, even if genetically modified. This has led many countries, among them Brazil, to establish

exceptions. For example, in Brazil living things (or parts thereof) are not patentable, except transgenic microorganisms (Art. 18, III). The majority of countries, however, follow the European example, where only varieties of plants and animals are not eligible for patent protection.

In Brazil, for instance, the recombinant form of molecules such as IFN-alpha and human erythropoietin can be patented, as well as the processes for obtaining them using animal cells. However, the natural forms of these molecules, as well as the animal cells used to obtain these molecules, are not patentable.

15.6 Industrial property and technology transfer offices

The search by the business sector for new sources of technology and the avidity of universities for new sources of funding have started to give a new face to some academic environments. Academic–business relations take many forms: investment of companies in complete projects or parts of projects, licensing for exploitation of patents, among others.

In whatever form, a company will be much more willing to fund research if there are guarantees that the technology will not wind up in the public domain, and will instead receive adequate patent protection. In the United States, where these movements began to gather steam in the 1970s, universities had to prepare themselves to deal with these new questions. On the other hand, the US Government found that the research financed with public funds often wound up on the ''shelves.'' This prompted the need for legislation, which culminated in 1980 with passage of the Bayh-Dole Act. This act authorizes universities and small companies to retain the patent rights that result from research funded by the federal government.

As an indication of the effect of the Bayh-Dole Act, before 1980 fewer than 250 patents a year were issued to American universities. In 2004, a survey conducted by the Association of University Technology Managers indicated that 4458 new licensing agreements were signed that year, and the number of patent applications filed by universities was 13 021. Between 1991 and 2004, the survey reported an increase of over 100% in patent filings, as well as a 142% jump in the number of licensing and option agreements signed. These option agreements permit a company to acquire the right to evaluate a technology for a given period, called the option period, before deciding whether or not to actually license it.

The survey also found, however, that universities and research institutions need to be patient and should not expect millionaire returns in short time-frames, since the generation of revenues from licensing is not immediate. Fewer than 1% of the 20 968 licenses active in 2000 generated a million dollars or more. Of the institutions responding to the survey, only 10% reported profit greater than 20 million dollars from licensing, and 80% of these licenses had already been in force for at least 10 years.

In academic environments, the establishment of industrial property and technology transfer offices has arisen from the increased activities to protect inventions, the need to transfer technologies to enable their production, and from legal requirements for obtaining patents, all driven

by the search for profits from research and development investments through commercialization of the technologies patented.

In Brazil, researchers have started to wake up to the need to patent their inventions, albeit tardily and slowly. The number of patent applications filed with the INPI (Brazilian PTO), even though small compared with the number of journal publications and conference proceedings, is increasing every year. Of the 22 000 applications filed in 2001, 6200 were by Brazilians. In 2000, Brazilians filed 5800 of the 20 000 applications with the INPI.

Besides seeking assistance from a qualified professional, researchers from some institutions in the country can call on specialized units at their institutions to support and guide them. Among the institutions that have established such units are Embrapa, Fiocruz, Unicamp (Campinas/SP), University of São Paulo, Minas Gerais Federal University, Rio de Janeiro Federal University, and Rio Grande do Sul Federal University, among others. The São Paulo State Research Support Foundation (Fapesp) maintains a Patent and Licensing Unit (Nuplitec) to guide researchers whose projects are financed by Fapesp.

In 2004, Law 10,973 was enacted, called the Law of Innovation, which among other provisions, requires public-funded scientific and technology institutions to have an innovation unit to encourage patenting and licensing of technology. This is an important instrument to protect the intellectual property generated at these institutions, as well as the negotiation for licensing and transfer to the market. Before negotiating with prospective partners, it is necessary to sign a confidentiality agreement between the parties to preserve the secrecy of the information exchanged during the negotiating phase. All this guidance and support is the responsibility of the internal intellectual property and technology transfer office.

According to Chamas (2001), although the debate and interest in intellectual property rights have arrived tardily in Brazil, the legal barriers are gradually being removed and the climate is becoming more receptive to the new activities. In this sense, the move by Brazilian universities is evident to protect and exploit their intellectual property. A portfolio of patents and similar assets should be the object of a competent business policy, including frequent analysis of the advisability of commercializing scientific ideas. The construction of an ample portfolio of intellectual property rights is justified to protect and reward scientific effort and allow a period of protected commercialization. Nevertheless, in the case of the public sector, this may not be the only motivation, since concern must be given to the country's social welfare and economic development.

Mention must also be made of the crucial role of support agencies for the structuring and consolidation of these internal offices.

An article by Kingston (2000) reports the history of the ''antibiotics revolution'' and how the discovery of Pasteur in 1877 that certain bacteria were able to inhibit the growth of anthrax organisms supplies important lessons for economic innovation. According to the article, questions such as the establishment of strategies for patenting and ownership of intellectual property, cooperation between universities and industries, and the role of support agencies must be considered when seeking successful innovation of a product/process.

The distinction between the profile of an inventor and innovator is quite clear from the report involving the invention and innovation of penicillin and streptomycin. According to Schumpeter (1988), an inventor produces ideas, while an innovator makes things happen, and materializes ideas. Personal commitment and willpower are characteristics of an innovator. This is the difference between Fleming and Florey in relation to the discovery and innovation of penicillin, respectively. The researcher/inventor has to deal with resistance to new ideas without the ability to make these new ideas accepted. In contrast, an innovator has the ability necessary to promote ideas and transform them into reality.

Researchers need to be capable of perceiving that an experiment may generate more information than it is possible to foresee before starting. On this point, Kingston (2000) shows the misleading effect that a partial success can have, which he exemplifies by the fact that Fleming was only able to perceive a limited use of his discovery of penicillin, allowing this partial success to mask the possibility of its use as an antibiotic.

This is where the innovator comes in, capable of noting this previously unidentified potential, and especially knowing how to use his or her personal characteristics to find the means to overcome difficulties and convince others to invest in developing and materializing the idea.

15.7 Patent and technology transfer specialists

An internal intellectual property and technology transfer office will have two types of professionals: the industrial property specialist and the technology transfer specialist. These activities require specific qualifications and extensive training, involving expertise normally not mastered by scientists. For this reason, the interactions between universities and research institutions on the one hand and companies on the other regarding patenting and marketing of inventions warrant specific handling.

The intellectual property specialist, usually with a professional background in engineering, chemistry, physics, or biomedical sciences, needs to have an understanding of international law, treaties, and conventions as well as national laws, decrees, edicts, etc., and the entire bureaucratic apparatus in the field. He or she must also interact with scientists to obtain the information necessary to draft the patent application, study the state of the art to prepare the diagrams, determine the scope of claims, see to the deposit of biological material with an international depositary authority, develop patenting strategies, deal with industrial property agents, follow the progress of the application, see to maintenance of patents already granted, monitor the patents and applications of competitors, contest negative opinions from patent offices, and respond to the various technical and legal demands involving the whole process, including any violation of intellectual property rights (Chamas and Müller, 1998).

Regarding monitoring patent applications of third parties, Brazilian legislation allows any interested party to submit documents and information during an application process period, to assist the technical examination. This is provided in Article 31 of the Industrial Property Law, and can be done up to the final examination. The tools for this are the Industrial

Property Review and the INPI site. Once a patent has been granted, no appeal is possible, but administrative cancellation can be sought within 6 months. Court action to nullify a patent can be taken at any time during its lifetime.

Technology transfer specialists, in contrast, are more focused on business aspects. They need to be able to work closely with and understand the work of the industrial property people, to help them check on the patent applications filed by the institution. But they also, and more importantly, must follow market trends involving the portfolio of patents and applications, orient the preparation of technical cooperation, detect and contact potential partners for future technology transfers, negotiate and draft contractual instruments applicable to each case, monitor the partnerships formed, deal with external law offices specializing in intellectual property, and act in cases of breach of contractual clauses. In relation to this last item, when a contractual infraction or other compliance problem is noted, a choice must be made as to what action to take. Will it be by judicial action or some form of alternative dispute resolution?

There is a range of alternative dispute resolution (ADR) methods. Among the most common are arbitration and mediation, although conciliation and direct negotiation are also possible.

Dispute resolution is an important process for business, since it permits a quick, fair, and economic way to solve conflicts, without the need for prolonged litigation. Hence, it is possible to obtain a more satisfactory outcome and often to preserve a suitable commercial relationship.

Some activities need to be carried out jointly by the industrial property and technology transfer specialists. An example is the decision on whether or not an invention is patentable, or should be patented, based on technical and economic considerations, and industrial property policies.

Some aspects of intellectual property that need to be evaluated by these specialists when analyzing a research project are listed below (Müller, 2003).

(i) Searching for prior art and verifying the satisfaction of patentability requirements.
(ii) Confirming with inventors the absence of disclosure of the result of the research, even in articles submitted for publication.
(iii) If the patentability requisites are met, preparing the first draft of the application from the technical information sent by the inventors.
(iv) Whenever possible, including functional equivalents of the invention to enlarge the scope of the protection.
(v) In parallel, confirming who the inventors are and to what companies/institutions they belong.
(vi) In the case of external inventors, verifying if there is a formal partnership agreement.
(vii) If so, verifying whether the agreement contains ownership clauses.
(viii) Identifying the percentage of intellectual contribution of each inventor in materializing the invention, information that will influence the percentage that will accrue to each company/institution.
(ix) Verifying whether there were or will be other external sources of

funding for the project besides the outlay of the company/institution itself.

(x) Whenever possible, negotiating the ownership of the company/institution and proposing an agreement between the parties stipulating the percentage that belongs to the other party or parties as a result of commercial exploitation of the invention.

15.8 Conclusions

Based on the above observations, the importance of patent protection of biotechnology inventions is clear, as well as the need for professionals trained to take care of the protection and licensing of the intellectual property generated by universities, research institutions, and companies.

Public–private partnerships should be encouraged as essential for the development of products and processes, and especially to leverage investment seeking to reduce the dependence on imported biopharmaceuticals. For this, it is vital that institutions adequately project their research results, to enable them to better attract investors to fund technological development. Hence, it will be possible to increase the chances that an invention will turn into an innovation.

References

Association of University Technology Managers (1999), AUTM Licensing Survey FY 1996. Norwalk: AUTM.

Beier FK, Straus J (1986), Genetic engineering and industrial property, In: The Sixteenth Bitburg Colloquium on Genetic Engineering and Law, Bitburg, Abstracts.

Bull AT, Holt G, Lilly MD (1982), Biotechnology: International Trends and Perspec tives, OECD, Paris.

Chamas CI (2001), Proteção e Exploração Econômica da Propriedade Intelectual em Universidades e Instituições de Pesquisa, COPPE/UFRJ, PhD Thesis.

Chamas CI, Müller ACA (1998), Gerência da Propriedade Industrial e da Transfer ência de Tecnologia, In: XX Simpósio de Gestão da Inovação Tecnológica, São Paulo, Abstracts.

Gama Cerqueira JDA (1982), Tratado da Propriedade Industrial, 2nd Ed., Editora Revista dos Tribunais, Rio de Janeiro.

Kingston W (2000), Antibiotics, invention and innovation, Research Policy 29: 679 710.

Müller ACA (2003), Patenteamento em Biotecnologia: Abrangência e Interpretação de Reivindicações, EQ/UFRJ, PhD Thesis.

Schumpeter JA (1988), A teoria do desenvolvimento econômico, Nova Cultural, São Paulo.

Recombinant therapeutic proteins

16

Maria Candida Maia Mellado and Leda dos Reis Castilho

16.1 Introduction

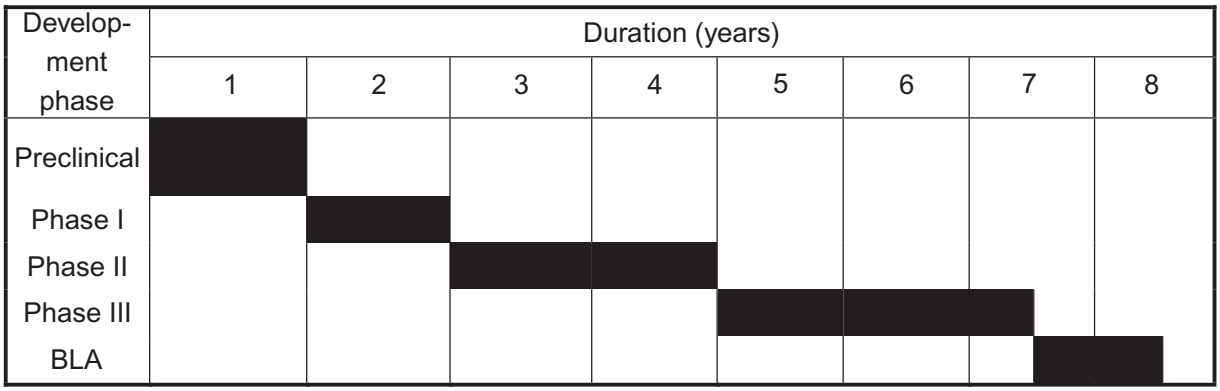
Biopharmaceuticals are proteins and nucleic acid-derived molecules that are used for therapeutic purposes or *in vivo* diagnostics, and are produced by means other than direct extraction from a native (non-engineered) biological source (Walsh, 2002). When considered together with vaccines and biomolecules extracted from natural sources, they are designated ''biologicals'' by the pharmaceutical industry.

A survey carried out by the Pharmaceutical Research and Manufacturers of America (PhRMA) verified that in 2004 over 100 biopharmaceuticals had already gained approval from the US Food and Drug Administration (FDA) (PhRMA, 2004). This survey found that 324 new biotechnology medicines, targeting nearly 150 diseases, were undergoing human clinical trials or under review by the FDA. Among the products under development, almost half (154) were intended for cancer treatment, and another significant portion for infectious diseases, AIDS/HIV and related conditions, and autoimmune and neurological disorders. Despite the long development time for biopharmaceuticals (*Figure 16.1*) and the fact that most molecules under clinical trials do not gain final approval from the regulatory agencies, a considerable increase in the number of commercialized biopharmaceuticals is expected in the next few years.

It is estimated that to date more than 250 million people worldwide have benefited from the biologicals already approved for the treatment or prophylaxis of cardiac diseases, multiple sclerosis, several types of cancer, hepatitis, arthritis, and diabetes, among others. Since biopharmaceuticals focus their action on the molecular basis of diseases, they are providing physicians and patients with new powerful tools for the treatment of diseases, changing fundamentally the way diseases are treated (PhRMA, 2004).

16.2 Main therapeutic proteins

Therapeutic proteins can be divided into seven different groups: cytokines, hematopoietic growth factors, growth factors, hormones, blood products, enzymes, and antibodies (Walsh, 2003). Most of these proteins have complex structures and undergo different post-translational modifications,



Phase	IND	Phase I	Phase II	Phase III	Commercial
Premises	Expression system defined Process scalability	Primary definition of process No validation	Refinement of operational control parameters Development of scale-down process models for validation Process out-of-limit definition	Finalization of process control parameters Fixed and defined process and products Pivotal process validation and characterization studies	Validated production process Well-characterized product Robust process

Figure 16.1

Phases in the development of a biopharmaceutical and the concept of time to market. IND, Investigational New Drug Application; BLA, Biologics License Application (adapted from Werner, 2004).

such as glycosylation, which usually is essential for biological activity, as discussed in Chapter 6. For this reason, most of the biopharmaceuticals already approved or under development are produced by mammalian cells, since microorganisms and insect cells are not able to correctly perform the required post-translational modifications. Among mammalian cells, the cell line most usually employed in the production of recombinant therapeutic proteins is the CHO cell line (Chinese hamster ovary cells), which provides proteins with a glycosylation pattern very similar to that of human native proteins. Besides, CHO cells are considered safe because the major viruses that cause disease in humans are not able to replicate in these cells. The manufacturing processes for these proteins are all based on principles discussed in the previous chapters of this book.

16.2.1 Cytokines

Cytokines play a central role in regulating the immune and inflammatory response. Interferons (IFNs) were the first family of cytokines to be

discovered, and are used as biopharmaceuticals to enhance the immune response against infectious agents (viruses, bacteria, and protozoa) and to treat some autoimmune conditions and some types of cancer (*Table 16.1*). There are distinct classes of IFNs, and humans produce at least three; IFNα, IFNβ, and IFNγ (Walsh, 2003).

The IFNα class consists of approximately 20 subtypes of molecules, most of them having 165–172 amino acids and a molar mass of approximately 20 kDa. Depending on the subtype, they can be either O-glycosylated or non-glycosylated, and present over 70% amino acid homology with each other. Beyond being abundant in leucine and glutamate, they are characterized by conserved cysteines, usually at positions 1, 29, 99, and 139, which generally form two disulfide bonds. The secondary structure is characterized by several α-helices and no β-sheets. The major application of IFNα as a biopharmaceutical is the treatment of hepatitis, but some commercial preparations have already been approved for leukemia and other types of cancer.

IFNβ is produced *in vivo* normally by fibroblasts. In humans, only one IFNβ is found, which has 166 amino acids and a molar mass larger than 20 kDa. The molecule has a disulfide bond as well as an N-linked carbohydrate chain bound to asparagine 80. Structurally, it is characterized by the presence of five α-helices. Recombinant IFNβ is marketed under the names of Betaferon®, Betaseron®, Avonex®, and Rebif®, being indicated for the treatment of multiple sclerosis, since it blocks the secretion of other cytokines involved in the pathogenesis of this disease.

IFNγ, also known as immune interferon, is produced *in vivo* predominantly by lymphocytes. Human IFNγ has 143 amino acids, with a molar mass varying between 17 and 25 kDa, depending on the level of N-glycosylation. The secondary structure consists of six α-helices. The most important therapeutic application of IFNγ is the treatment of chronic granulomatous disease (CGD), a rare genetic condition characterized by the deficiency of phagocytic cells. CGD is characterized by repeated infections in sufferers because of the absence of phagocytes to ingest or destroy infectious agents (Walsh, 2003).

Until the 1970s, the source of exogenous IFN for therapeutic use was human blood itself. However, recombinant DNA technology made possible the cloning of IFN genes into several expression systems, such as *Escherichia coli*, yeast, and mammalian cells, facilitating the large-scale production of these proteins and increasing safety. The level of glycosylation of these proteins determines the expression system in which they should be expressed. IFNα, for example, is not glycosylated in its native form and therefore can be expressed in *E. coli*. Mammalian cells, especially CHO cells, may be used to produce the other IFN classes, which are glycosylated.

Another cytokine class used as a biopharmaceutical is interleukin (IL), which consists of at least 25 different subtypes (IL-1 to IL-25). Except for IL-1, most interleukins are glycosylated and have a molar mass in the range of 15–30 kDa. IL-2 is the most well studied interleukin, and its recombinant form is approved for the treatment of renal cell carcinoma. Since the absence of glycosylation does not affect its biological activity, rIL-2 is produced in genetically engineered *E. coli*.

16.2.2 Hematopoietic growth factors

Hematopoietic growth factors are also cytokines and play an essential role in the formation and differentiation of blood cells (hematopoiesis). The main molecules of this family are erythropoietin (EPO), granulocyte colony-stimulating factor (G-CSF), macrophage colony-stimulating factor (M-CSF) and granulocyte-macrophage colony-stimulating factor (GM-CSF).

GM-CSF, also known as pluripoietin-α, is a glycosylated polypeptide with 127 amino acids and a molar mass of approximately 22 kDa. The secondary structure exhibits four α-helices and two anti-parallel β-sheets. G-CSF (or pluripoietin) is a glycoprotein with two splicing variants, the predominant one presenting 174 amino acids and 21 kDa, with only one O-glycosylation site. Its structure is characterized by the presence of two disulfide bonds and four α-helices. M-CSF (or CSF-1) has three N-glycosylation sites and several disulfide linkages. There are three forms of this protein, with molar masses varying from 45 to 90 kDa and the largest one having 522 amino acids (Walsh, 2003).

The three types of recombinant CSFs (GM-CSF, G-CSF, and M-CSF) are used as hematopoietic stimulators, in the treatment of infectious diseases, some types of cancer, bone marrow transplants, and neutropenia, a disease characterized by a reduced level of neutrophils, the precursors of white blood cells (*Table 16.1*).

EPO is a globular glycoprotein, characterized by four α-helices and two anti-parallel β-sheets, which contains 166 amino acids. It has a molar mass between 34 and 40 kDa, 40% of which is due to carbohydrate chains. The molecule has three N-glycosylation sites (asparagine residues 24, 38, and 83) and an O-glycosylation site (serine 126). The carbohydrate chains contribute to the solubility, *in vivo* metabolism, and cellular processing of the molecule. However, its complex glycosylated tetra-antennary structure and the high degree of sialylation enhance the importance of a careful control of operational conditions during the manufacturing process. This aims at minimizing the micro- and macroheterogeneity and, consequently, maximizing the yield in terms of isoforms with high biological activity (see Chapter 6).

The first approval of a therapeutic use for recombinant EPO was in 1989 for the treatment of anemia related to chronic renal failure. The treatment with EPO stimulates production of erythrocytes and improves the patient's quality of life, as well as reducing or eliminating the need for blood transfusion. There are other non-renal applications, such as the minimization of blood transfusion after surgery, the prevention of anemia after bone marrow transplantation, and the treatment of anemia caused by the use of antiretroviral drugs, by chemotherapy, and by prematurity.

16.2.3 Growth factors

Apart from the hematopoietic growth factors, discussed in Section 16.2.2 above, there are other types of growth factors, such as transforming growth factor (TGF), platelet-derived growth factor (PDGF), insulin-like growth factor (IGF), and epidermal growth factor (EGF).

TGF consists of a family of growth factors employed in the treatment of bone fractures and skin ulcers. TGF molecules of type β exist in the form of homodimers of 112 amino acids. Two members of this family have their recombinant form approved for treating tibia fractures (Osigraft® and InductOs™), being produced in mammalian cells (*Table 16.1*).

PDGF, which plays an important role in wound healing (cicatrization), is a dimer formed by two polypeptides. There are two isoforms of this protein that are most commonly encountered in the human body: one with 110 amino acids and another with 125. Both isoforms have one glycosylation site and three disulfide bonds.

IGF is a polypeptide of 70 amino acids, with three disulfide bonds and a molar mass of 7.6 kDa. Its therapeutic use is still under study and future medical applications for this protein are likely to be the treatment of dwarfism, diabetes type 2, and renal diseases, among others.

EGF has a sequence of 1208 amino acids divided into seven domains, the largest being a glycosylated peptide of 53 amino acids. The secondary structure is characterized by the presence of two anti-parallel β-sheets and three disulfide bonds, which confer stability to the molecule. This protein is indicated for the treatment of wounds and skin ulcers.

16.2.4 Hormones

Hormones are proteins responsible for regulating molecular synthesis *in vivo*. The most well known therapeutic hormones are insulin, glucagon, growth hormones, and gonadotropins.

Recombinant human insulin and recombinant human growth hormone, both produced by microbial cells, were the first biopharmaceuticals to obtain approval from the regulatory agencies. Insulin is widely used for diabetes treatment, whereas growth hormone is used in the treatment of short stature, obesity, and for stimulating ovulation.

The gonatropins are a family of hormones that include follicle stimulating hormone (FSH), luteinizing hormone (LH), and chorionic gonadotropin (CG), among others. These three proteins are heterodimers that contain an identical polypeptide subunit (α) and another specific subunit (β), which confer the respective biological activity. FSH has a molar mass of 34 kDa, 14% of it being due to carbohydrate side chains. LH has a molar mass of 28.5 kDa.

The recombinant forms of these proteins (Gonal-f®, Luveris®, Ovitrelle®, and Puregon®), produced in CHO cells, are indicated for treating female infertility (*Table 16.1*).

16.2.5 Therapeutic enzymes

There is a great range of recombinant enzymes used for therapeutic purposes, but not all of them are expressed in mammalian cells.

Tissue plasminogen activator (tPA) is a protease with 527 amino acids and 4 glycosylation sites that acts *in vivo* as a thrombolytic agent. Its function is to proteolytically convert the zymogen, plasminogen, into active plasmin, which in turn degrades fibrin strands, thus dissolving the clots (Walsh, 2003). For this reason, recombinant tPA molecules

Table 16.1 Examples of biopharmaceuticals produced in mammalian cells approved for commercialization

Classification	Product name	Protein	Indication
Cytokines	Avonex®	Interferon β1a	Multiple sclerosis
	Rebif®	Interferon β1a	Multiple sclerosis
Hematopoietic growth factors	Epogen® /Procrit	Epoetin α	Anemia
	Epogin/Recormon	Epoetin β	Anemia
	Neorecormon®	Epoetin β	Anemia
	Nespo/Aranesp®	Darbepoetin α	Anemia
	Neutrogin® /Granocyte	G CSF (lenograstim)	Neutropenia
Hormones	Gonal f®	FSH	Female infertility
	Luveris® /Lutropin alfa	Luteinizing hormone	Female infertility
	Ovitrelle® /Ovidrel/ Choriogonadotropin α	Gonadotropin	Female infertility
	Puregon® /Follistim AQ/Follitropin β	FSH	Female infertility
	Thyrogen®	Thyrotropin α	Thyroid function (*in vivo* diagnostics)
Growth factors	InductOS® /Dibotermin α	Bone morphogenetic protein 2	Tibia fracture, spinal surgery
	Osigraft® /Eptotermin α	Bone morphogenetic protein	Tibia fracture
Blood coagulation factors	Advate	Factor VIII	Hemophilia A
	BeneFIX	Factor IX	Hemophilia B
	Helixate® NexGen/ Kogenate® FS	Factor VIII	Hemophilia A
	Kogenate®	Factor VIII	Hemophilia A
	NovoSeven®	Factor VIIa	Hemophilia A and B
	Recombinate	Factor VIII	Hemophilia A
	Refacto®	B domain deleted factor VIII	Hemophilia A
Therapeutic enzymes	Activase® /Alteplase	tPA	Acute myocardial infarction
	Cerezyme®	Glucocerebrosidase	Gaucher's disease
	Fabrazyme® /Agalsidase β	α Galactosidase A	Fabry disease
	Hylenex™	Hyaluronidase	Increase absorption and dispersion of other injected drugs
	Myozyme® /Alglucosidase α	Acid α glucosidase	Pompe disease
	Naglazyme™/Galsulfase	N Acetylgalactosamine 4 sulfatase	Mucopolysaccharidosis VI
	Pulmozyme®	DNAse I	Cystic fibrosis
	Replagal® /Agalsidase α	α Galactosidase	Fabry disease
	TNKase/Tenecteplase™/ Metalyse	tPA	Acute myocardial infarction
	Xigris® /Drotrecogin α	Activated protein C	Severe sepsis

Cell line	Manufacturing process data	First approval date	Company	Countries where approved
CHO	Bioreactor	1996	Biogen	Europe, USA
CHO	Bioreactor with microcarriers	1998	Serono	Europe, USA
CHO	Roller bottles	1989	Amgen	Several
CHO		1990	Chugai	Japan, Europe
CHO		1997	Roche	Europe
CHO		2001	Amgen	Europe, USA
CHO		1991	Chugai	Japan, Europe
CHO		1995	Serono	Europe, USA
CHO	Bioreactor	2000	Serono	Europe, USA
CHO		2000	Serono	Europe, USA
CHO	Bioreactor	1996	Organon	Europe, USA
CHO	Repeated batches	1998	Genzyme	Europe, USA
CHO	Suspension bioreactor	2002	Wyeth	Europe
CHO		2001	Howmedica International	Europe
CHO	2500 L bioreactor	2003	Baxter	Europe, USA
CHO	Fed batch bioreactor	1997	Genetics Institute	Europe, USA
BHK	Perfusion bioreactor	2000	Bayer	USA, Australia, Europe, Switzerland
BHK	Perfusion bioreactor	1993	Bayer	Several
BHK	Bioreactor, repeated batches	1996	Novo Nordisk	Europe, USA, Switzerland
CHO		1992	Baxter	Several
CHO	Perfusion bioreactor	1999	Genetics Institute/Biovitrum AB	Europe, USA
CHO		1986	Genentech	Several
CHO		1994	Genzyme	Europe, USA
CHO	Continuous bioreactor	2001	Genzyme	Europe, USA
CHO		2005	Halozyme Therap.	USA
CHO		2006	Genzyme	Europe, USA
CHO		2005	BioMarin	Europe, USA
CHO		1993	Genetech	Sweden, USA, Switzerland
Human fibroblasts		2001	Shire Human Genetic Therapies	Europe
CHO	12 000 L bioreactor	2000	Genentech/Boehringer Ingelheim	Europe, USA
HEK 293	Bioreactor	2001	Eli Lilly	Europe, USA

Continued

Table 16.1 *Continued*

Classification	Product name	Protein	Indication
Fusion proteins or immuno conjugates	Amevive/Alefacept	Fusion protein (IgG1/LFA3)	Psoriasis
	Enbrel® /Etanercept	Fusion protein (IgG1/TNFR)	Rheumatoid arthritis
	Myoscint® /Imciromab Pentetate	Immunoconjugate (IgG2a/DTPA)	Cardiac imaging (*in vivo* diagnostics)
	ProstaScint® /Capromab Pendetide	Immunoconjugate (IgG1/DTPA)	Prostate *in vivo* diagnostics
	Zevalin® /Ibritumomab Tiuxetan	Immunoconjugate (IgG1/MX)	NonHodgkin's lymphoma
Antibodies	Avastin® /Bevacizumab	Immunoglobulin G1	Colorectal cancer
	Bexxar® /Tositumomab	Immunoglobulin G2a	NonHodgkin's lymphoma
	Campath® / MabCampath/ Alemtuzumab	Immunoglobulin G1	Chronic lymphocytic leukemia
	Erbitux® /Cetuximab	Immunoglobulin G1	Head, neck, and colorectal cancer
	Herceptin® /Trastuzumab	Immunoglobulin G1	Breast cancer
	Humira® /Adalimumab	Immunoglobulin G1	Rheumatoid arthritis
	Panorex®	Immunoglobulin G2a	Colorectal cancer
	Raptiva® /Efalizumab	Immunoglobulin G1	Psoriasis
	Remicade® /Infliximab	Immunoglobulin G1	Rheumatoid arthritis, Crohn's disease, among others
	ReoPro® /Abciximab	Immunoglobulin G (fragment)	Ischemic cardiac complications
	Rituxan® /MabThera	Immunoglobulin G1	NonHodgkin's lymphoma, rheumatoid arthritis
	Simulect® /Basiliximab	Immunoglobulin G1	Prophylaxis of organ rejection in renal transplantation
	Synagis® /Palivizumab	Immunoglobulin G1	Prevention of respiratory tract diseases
	Xolair® /Omalizumab	Immunoglobulin G1	Asthma
	Zenapax® /Daclizumab	Immunoglobulin G1	Prophylaxis of organ rejection in renal transplantation
Others	GenHevac B Pasteur®	HBsAg	Hepatitis

Data from: European Medicines Agency (2006); Food and Drug Administration (2006); Castilho & Medronho (2002).

Cell line	Manufacturing process data	First approval date	Company	Countries where approved
CHO		2003	Biogen	USA
CHO		1998	Immunex/Wyeth	Europe, USA
Hybrid oma		1989	Centocor	Europe, USA
Hybrid oma	Hollow fiber bioreactor	1996	Cytogen	USA
CHO		2002	Idec	Europe, USA
CHO		2004	Genentech/Roche	Europe, USA
Hybrid oma		2003	Corixa/GlaxoSmithkline	USA
CHO	Suspension bioreactor	2001	ILEX/Genzyme	Europe, USA
SP2/0	10 000L batch bioreactor	2004	ImClone/Merck	Europe, USA
CHO	12 000 L batch bioreactor	1998	Genentech/Roche	Europe, USA
CHO	Batch bioreactor	2002	Abbott Laboratories	Europe, USA
		1995	Glaxo	Germany
CHO		2003	Genentech/Serono	Europe, USA
SP2/0	Perfusion bioreactor	1998	Centocor	Europe, USA
SP2/0	Perfusion bioreactor	1997	Centocor	USA
CHO	Suspension bioreactor	1997	Idec/Genentech/Roche	Europe, USA
Murine myeloma		1998	Novartis	Europe, USA
NS0	Fed batch bioreactor	1998	MedImmune/Abbott	Europe, USA
CHO	Suspension bioreactor	2003	Genentech/Novartis	Europe, USA
NS0	Suspension bioreactor	1997	Hoffmann La Roche	Europe, USA
CHO		1989	Pasteur Mérieux	France

(Alteplase and Tenecteplase™) are used in the treatment of acute myocardial infarction.

Urokinase is also a thrombolytic agent, used for treating pulmonary embolism. Two variants of this protease have already been isolated: one of 54 kDa and another of 33 kDa, both displaying proteolytic activity over plasminogen. Until recently, the only exogenous source for this enzyme was urine. However, in 2002 the product called Abbokinase®, which is produced in neonatal kidney tissue culture, was approved in the USA.

Gaucher's disease is a disorder of genetic origin, characterized by the absence of a vital enzyme called glucocerebrosidase. In this case the glucocerebroside, the lipid component of cell membranes, accumulates in the organism, leading to an exaggerated enlargement of the liver and other organs. Cerezyme®, produced by the company Genzyme, is the recombinant version of glucocerebrosidase, expressed in CHO cells (*Table 16.1*).

Another genetic metabolic disorder is Fabry disease, which is related to a deficiency in the enzyme α-galactosidase A. This enzyme is involved in the metabolism of lipids, and its deficiency leads to the accumulation of lipids in the eyes, kidneys, and nervous and cardiovascular systems. In 2001, the biopharmaceutical called Fabrazyme® was approved, which consists of recombinant α-galactosidase A and is produced by genetically modified CHO cells.

16.2.6 Blood coagulation factors

There are 13 factors involved in the blood coagulation process. Some of them, such as factors VII, VIII, and IX, are proteins. The absence of these proteins constitutes a genetic disease known as hemophilia. A hemophilic person is constantly at risk of undergoing a hemorrhage because of the incapacity to correctly carry out coagulation. Until the development of recombinant blood coagulation factors in the 1990s, hemophiliacs were treated by blood transfusion and with proteins obtained from the plasma of human donors. However, both solutions presented high risk of transmission of pathogenic agents, especially viruses. This is why a significant number of persons affected with hemophilia were contaminated with HIV and hepatitis viruses in the 1980s. Since then, although an additional virus inactivation step has been added to the manufacturing of plasma-derived molecules to increase product safety, there is still the risk of transmitting unknown viruses and pathogens of other types. Apart from that, limitations related to the availability of raw material (plasma from donors) also pose problems for plasma-derived coagulation factors. For these reasons, an increasing demand for recombinant coagulation factors has occurred in recent years and, in some countries such as Canada and Ireland, the exclusive use of recombinant factors is obligatory.

Blood factor VIII (FVIII) is a glycoprotein with 2351 amino acids and 330 kDa. Its deficiency causes hemophilia A. The first products based on recombinant factor VIII to reach the market were Recombinate and Kogenate®, expressed in CHO and BHK cells, respectively. Over the last decade, other rFVIII products were approved, with modifications to the molecule (e.g. deletion of the B-domain), in the formulation or in the production processes.

In the production process of Kogenate®, BHK cells are cultivated in perfusion bioreactors up to 500 L in size. The first step of purification involves anion exchange chromatography. After that, an immunoaffinity chromatographic step and gel filtration are carried out, followed by another immunoaffinity and another anion exchange chromatography step (Boedeker, 1992; Bhattacharyya *et al*., 2003).

Recombinate is produced in CHO cells cultivated in 2500 L bioreactors, operated in fed-batch mode. The purification process starts by removing cells by a filtration step, followed by three chromatographic steps: immunoaffinity, anion exchange, and cation exchange (Bhattacharyya *et al*., 2003).

When hemophilia is due to a deficiency in factor IX (FIX), it is designated hemophilia B. In the same way as hemophilia A, the alternative to treatment based on blood transfusion or plasma-derived products is the use of recombinant FIX, which was approved for commercialization in 1997 with the name of BeneFix®.

It is produced in recombinant CHO cells cultured in a medium free of animal-derived components. The BeneFix® production process involves an ultrafiltration/diafiltration step, followed by four chromatographic steps: ion exchange (Q resin), pseudo-affinity (Cellufine sulfate resin), hydroxyapatite, and affinity (immobilized Cu^{2+} ions). After these chromatographic steps, there are membrane processes (nanofiltration for viral clearance and diafiltration for solvent exchange), after which the purified protein is formulated (Edwards and Kirby, 1999).

Some hemophilia A and B patients develop antibodies against FVIII and FIX, respectively. This complicates the direct administration of these proteins. An alternative in these cases is treatment with active factor VII (FVIIa), which complexes with factor III in the presence of phospholipids and Ca^{2+}, activating factor X, which, in normal patients, is activated by active factors VIII and IX. The commercial name of recombinant FVIIa expressed in BHK cells and produced by Novo-Nordisk is NovoSeven® (*Table 16.1*).

16.2.7 Antibodies

Therapeutic antibodies constitute the most studied and commercialized class of biopharmaceuticals nowadays. Their applications range from *in vitro* and *in vivo* diagnostics, to the treatment of cancer, cardiovascular diseases, asthma, and infectious and autoimmune diseases.

In the case of tumor treatment, in most cases, the mechanism of action of a monoclonal antibody (mAb) is based on the specific recognition and binding to specific surface receptors that are found on the tumor cells. However, in 2004, the antibody Avastin® (Bevacizumab), which acts as an inhibitor of the vascular endothelial growth factor (VEGF), was approved for the treatment of metastatic colorectal cancer. In this way, Avastin® inhibits angiogenesis around tumors, avoiding the abnormal formation of blood vessels that support tumor expansion. Additionally, mAbs can be conjugated to drugs, to direct these molecules to the target tumor cells, decreasing any adverse effects on normal cells.

In the case of cardiovascular diseases, clot formation in the vascular system not only interrupts blood flow but also leads to embolism with fatal consequences. Antibodies against fibrin are able to detect these clots in their initial stage of formation.

In viral infectious diseases, antibodies are used to bind to viral surface antigens, inhibiting disease propagation. Among the viruses most widely studied as potential antibody targets are the hepatitis B and HIV viruses.

One of the worst consequences of a bacterial infection is sepsis. Because of the release of bacterial endotoxins (lipopolysaccharides), the immune system acts to produce cytokines, which are only efficient for localized infections. However, in the case of septicemia, the excessive release of cytokines leads to dilatation of blood vessels, causing a drop in blood pressure and insufficient tissue irrigation. Antibodies against endotoxins can be used to minimize this condition.

Another application of antibodies is to treat autoimmune diseases, which occur due to a deficiency of the immune system that recognizes an endogenous molecule as a foreign element. The most common immunotherapeutic approach in these cases is that the antibody is directed against the antigen on the surface of the lymphocytes responsible for the immune response.

Finally, some antibodies are used in prophylaxis to prevent the rejection of transplanted organs, such as kidney, liver, and heart. An example is Zenapax®, which was approved for use in 1997.

Some examples of therapeutic mAbs are presented in *Table 16.1*. However, because of the clinical and commercial importance of this class of therapeutic proteins, an entire chapter is dedicated to monoclonal antibodies (Chapter 17).

16.3 Economic aspects

The biopharmaceutical global market grew from 2000 to 2004 at an annual rate of 19%, and was evaluated at US$ 48 billion in 2005. This represents a much higher growth rate than that experienced by the pharmaceutical industry as a whole. The forecast is that the biopharmaceutical market will reach US$ 100 billion by 2010 (Research and Markets, 2005a).

Erythropoietin (Epoetin, EPO) had a global market of US$ 11.1 billion in 2004 (Research and Markets, 2005b) and was the top-selling biopharmaceutical worldwide. The patent on this biopharmaceutical, owned by the company Amgen, expired in 2004, paving the way for new companies to enter the market.

The colony stimulating factors (G-CSF, GM-CSF, and M-CSF) are also commercially relevant. The annual sales growth rate of these proteins in the period 2000–2004 was 16% and in 2004 the sales totalled US$ 3.6 billion (Research and Markets, 2005c).

However, the most promising class of biopharmaceuticals consists of the therapeutic mAbs. In 2004, total sales of therapeutic mAbs reached approximately US$ 11.2 billion, with an impressive annual growth rate of 42% in the period 2000–2004 (Research and Markets, 2005d). The forecast is that in 2010 this market will reach US$ 34 billion.

According to Reichert and Pavlou (2004), 17 therapeutic mAbs, grouped into 4 different types, had been approved by the FDA at that time: 3 murine, 5 chimeric, 8 humanized, and 1 human. Among them, Remicade® / Infliximab was the top-selling one (US$ 1.6 billion in 2002), representing 30.5% of mAb sales in that year.

It is forecast that up to the end of the current decade, the research focus regarding therapeutic mAbs will be on two categories: oncology and arthritis, and inflammatory and immune disorders. A significant increase of sales is expected up to 2008 for chimeric mAbs, in absolute terms, and for human and humanized mAbs, in relative terms (*Figure 16.2*). Up to now, murine mAbs are those with the lowest approval rate (4.5%), whereas the chimeric are the most successful (26%). The approval rates for humanized mAbs (18%) and human antibodies (14%) were intermediate. However, as most of the last two types are still under development, the approval situation may change significantly in the future (Reichert and Pavlou, 2004).

This rapidly growing market is very competitive. Companies in this area must be highly innovative to be successful. There are key determinant factors that should be optimized for competitive innovation: (a) product development should be shortened by accelerating clinical trials; (b) well-protected intellectual property should be built and maintained; (c) the efficacy of products should be increased, for example, through new formulations, conjugation, or pegylation; (d) drug delivery should be

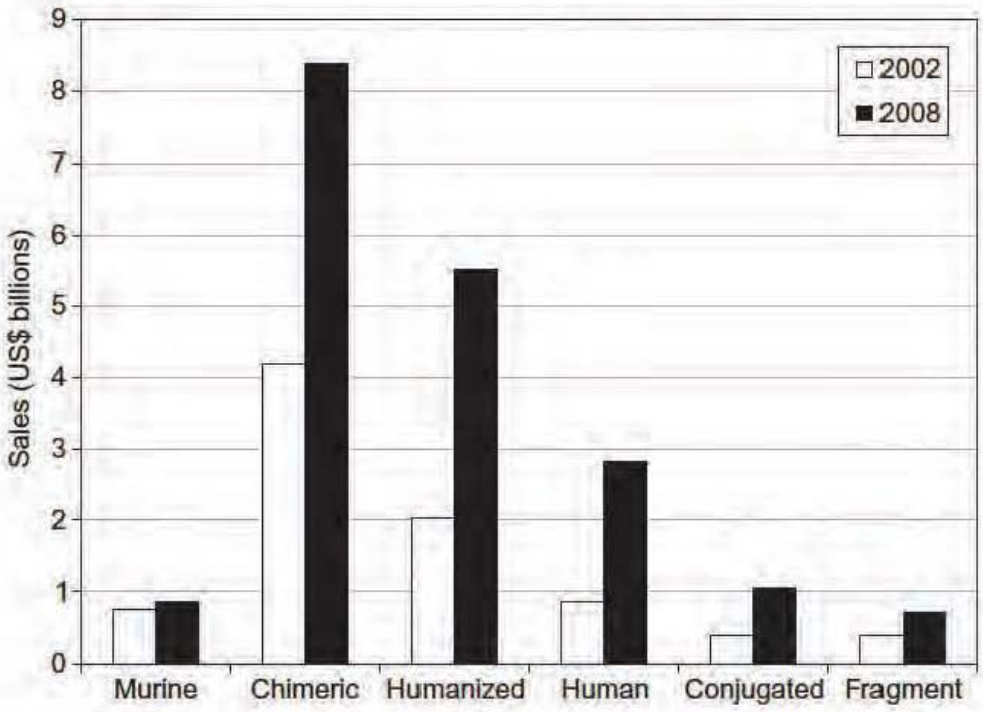


Figure 16.2

Comparison of sales of therapeutic monoclonal antibodies (mAbs) in 2002 (in white) and forecast for 2008 (in black) (adapted from Reichert and Pavlou, 2004).

improved; and (e) cost-efficient manufacturing capabilities should be developed (Pavlou and Belsey, 2005).

Cost-effective technologies have been gaining importance in the last few years due to the expiration of patents of the first-generation biopharmaceuticals (*Table 16.2*). In the past, the concept of ''time-to-market'' dominated the industrial arena. Nowadays, companies are willing to focus on process optimization and cost reduction. Important tools for this purpose are the use of advanced genetic manipulation techniques to increase the cell specific productivity, as well as the development of perfusion processes, to increase volumetric productivity, and more efficient purification processes, to improve yield (see Chapters 3, 9, 11 and 12).

16.4 Challenges and future perspectives

16.4.1 Formulation and delivery of biopharmaceuticals

Despite the significant development in the biopharmaceutical area in recent years, many challenges have yet to be overcome. Among the main obstacles faced by companies are the development of improved formulations and new routes of administration, as well as the delivery of the active molecules to the site of therapeutic action. Many biopharmaceuticals present limitations of low stability *in vitro* (shelf-life) or *in vivo* (half-life after injection in the patient), and low solubility and bioavailability (Muller and Keck, 2004).

Inhalation/nebulization is an alternative method of administration of some biopharmaceuticals, for example insulin and GM-CSF (Anderson

Table 16.2 Examples of biopharmaceuticals that have lost patent protection

Company	Commercial name	Protein	Year of patent expiration
Produced in animal cells			
Biogen	Avonex®	Interferon β 1a	2003
Amgen/J&J/Sankyo	Epogen®/Procrit	Epoetin α (erythropoietin)	2004
Genentech	Activase®	Alteplase (tPA)	2005
Genetics Institute	Recombinate	Factor VIII	2005
Genetics Institute/Wyeth	Benefix®	Factor IX	2005
Eli Lilly	Xigris®	Drotrecogin α (activated protein C)	2005
Produced in microorganisms			
Schering Plough	Intron A®	Interferon α 2a	2002
Novo Nordisk	Novolin®	Insulin	2002
Eli Lilly	Humulin®	Insulin	2002
Eli Lilly	Humatrope®	Human growth factor	2003
Genentech	Nutropin®	Human growth factor	2003
Genentech	Protropin®	Human growth factor	2004
Amgen	Neupogen®	Filgrastim (G CSF)	2006

Adapted from Dudzinski (2004).

et al., 1999). The first biopharmaceutical administered by inhalation to be commercialized is Exubera®, a powder insulin, which was approved by FDA and EMEA in 2006 (European Medicines Agency, 2006; Food and Drug Administration, 2006). This is the first alternative route of administration since the discovery of insulin in the 1920s.

Another possible form of administration that is under study is through the use of nanoparticles with diameters in the range of 200–400 nm, obtained through the formation of nanocrystals or by creating nanoscale structures that capture the biomolecules. Depending on the materials employed and the preparation method, distinct particles can be used: nanoparticles, liposomes, polymeric micelles, ceramic nanoparticles, and dendrimers.

Nanocrystals can increase *in vitro* stability by transforming soluble molecules into non-soluble forms. In this way, only the nanocrystal surface is accessible to degrading factors, such as water and oxygen. This means that an external monolayer of degraded molecules is formed to protect the inner part of the nanocrystal. Soluble molecules, such as peptides, nucleotides, and proteins can be transformed into particles by dispersing them into oil. After oral administration, the oil is degraded by lipases *in vivo*, releasing the drug. Alternatively, soluble molecules can be transformed into non-soluble molecules by forming lipid conjugates (LDC®). Conjugated particles can be prepared by either salt formation (e.g. amino group containing molecule with fatty acid) or covalent bond (e.g. ether, ester) (Muller and Keck, 2004).

Another challenge is the delivery of a biopharmaceutical to its site of action, as the injection of molecules in solution leads to a partitioning of the molecules according to their physicochemical properties. One approach to deliver particles injected intravenously is based on the concept of ''differential protein adsorption.'' After injection the particles adsorb blood proteins according to physicochemical surface properties of the particles. The adsorbed proteins determine the cells to which the particles will be directed (Muller and Keck, 2004).

A popular approach to confer enhanced stability and improve the pharmacokinetics of therapeutic proteins is to conjugate them to different polymers. Nowadays polyethylene glycol (PEG) is the most widely employed polymer due to its low toxicity and cost. This technique, known as pegylation, is able to increase protein stability, improve pharmacokinetics, and potentially decrease immunogenicity (Chirino and Mire-Sluis, 2004). Since the entry of the first pegylated protein in the market in 1990, several products have been approved, such as Neulasta® (pegylated G-CSF for the treatment of neutropenia induced by chemotherapy) and PEG-Intron™/Pegasys (IFNα for the treatment of chronic hepatitis C). These products have enhanced stability, thus reducing the number of injections patients need to receive. However, the companies owning the patents commercialize pegylated biopharmaceuticals at prices much higher than the corresponding nonpegylated ones, preventing a broader use of the pegylated version, mainly in developing countries. The prices charged certainly do not represent the additional cost of polymer conjugation, but rather a market value that reflects a monopoly.

16.4.2 Characterization of biopharmaceuticals

Biopharmaceuticals have completely different properties from conventional low molar mass pharmaceuticals. This particularly affects product characterization, due to the complex production processes and protein structures. Thus, successful production of biopharmaceuticals relies mainly on strict protocols, clinical expertise, and follow-up during clinical application (Crommelin *et al*., 2003).

For these reasons, the ability to compare products originating from different processes is more important than characterizing a biopharmaceutical product from a well established process. Modifications in the production process can result in changes in physicochemical properties and post-translational modifications. These modifications can influence biological activity, bioavailability, and immunogenicity of the product (Chirino and Mire-Sluis, 2004). Therefore, as discussed in Chapter 14, the regulatory agencies worldwide are discussing criteria to be applied to evaluate comparability of biological products (Food and Drug Administration, 2005; European Medicines Agency, 2005).

Among the many tests available for characterizing biopharmaceuticals, assessment of protein structure, particularly quaternary structure, including aggregate formation, seems to be particularly important, since it can strongly influence immunogenicity. This remains a concern for the characterization of biological products, since preclinical studies, carried out in animals, are not able to predict the immunogenicity of a biological drug in the target population (Chirino and Mire-Sluis, 2004). In this context, product labelling becomes an important issue, since aggregate formation and continuous administration of a protein drug can lead to antibody production (Ryff and Schellekens, 2002). Biopharmaceutical labelling could thus provide information in such a way that the physician would understand the clinical and biological consequences of antibody production and, therefore, could make a decision that could lead to the suspension of a treatment.

16.4.3 Alternative expression systems

Due to the structural complexity of therapeutic proteins, their recombinant forms are usually produced in mammalian cell culture. When glycosylation is not present or is not essential for biological activity, therapeutic proteins can be produced in bacteria (e.g. *E. coli*) or yeast (e.g. *Saccharomyces cerevisiae*). However, some studies propose the use of these systems for glycosylated proteins as well.

Baneyx and Mujacic (2004) propose the genetic manipulation of *E. coli*, to enable metabolic competency to carry out post-translational modifications. Gerngross (2004) argues for the use of filamentous fungi and yeast for the production of therapeutic proteins by the expression of human glycosylation enzymes. This type of strategy was reported by Chiba *et al*. (1998) and Martinet *et al*. (1998) for *S. cerevisiae* and *Pichia pastoris*, respectively.

Plant cells are another expression system, which is being evaluated for the production of recombinant therapeutic proteins. According to Hellwig

et al. (2004), recombinant human glycoproteins synthesized in plant cells show greater similarity to the native proteins than those produced in recombinant microorganisms. However, there are some limitations, such as the instability of recombinant proteins in plant cell culture media and the low protein yields reported so far for plant cells in culture (Hellwig *et al*., 2004). Among therapeutic proteins, GM-CSF has been produced in *Nicotiana tabacum* (Lee and Kim, 2002) and *Lycopersicum esculentum* (Kwon *et al*., 2003). A concrete example of the application of plant cells is the first market license issued in January 2006 to a veterinary vaccine produced in plant cells (Katsnelson *et al*., 2006). The North American USDA (United States Department of Agriculture) considered the veterinary viral vaccine safe and effective in protecting chickens from Newcastle disease. This subunit vaccine consists of a protein expressed in recombinant tobacco cells and represents the first vaccine obtained from plant cells to be approved worldwide.

Another possible system for the production of therapeutic proteins consists of transgenic animals. Although there is controversy regarding safety and reproducibility, many reports on this subject have been published and some proteins derived from these systems are currently under clinical trials. Parker *et al*. (2004) purified and characterized human fetoprotein α secreted in transgenic goat milk. This protein, used in the treatment of autoimmune diseases, proved to be identical to the native form by mass spectrometry, circular dichroism, and pharmacokinetic tests.

Using a different approach, some authors propose post-translational modification *in vitro.* Glycosylated proteins could be obtained by *in vitro* glycosylation after their production in non-mammalian expression systems. Synthetic strategies for preparing glycoproteins can be classified into two main categories: convergent and sequential. In the convergent strategy, the glycan is assembled separately by enzymatic or chemical methods, or obtained from natural sources, and is then attached to the polypeptide chain. In the sequential strategy, a mono- or disaccharide unit is introduced into the polypeptide, and then the complete glycan is synthesized by the sequential attachment of sugars by the action of glycosyltransferases. However, the development of simple and reliable methods for obtaining selectively and uniformly glycosylated proteins is still a challenge and will probably remain a focus of future research (Khmelnitsky, 2004).

Finally, some authors propose the use of enzyme mixtures for the biosynthesis of complex molecules. A cell free protein synthesis (CFPS) system is a novel approach that was successfully used for the production of complex mammalian proteins with multiple disulfide bonds (Bhattacharya, 2004). However, even if the technical potential of this method is proved, its economical feasibility has still to be proven.

16.4.4 Second-generation biopharmaceuticals

Most biopharmaceuticals initially approved, known as first-generation biopharmaceuticals, consist of simple replacement proteins with an amino acid sequence identical to the native human protein. Second-generation biopharmaceuticals are engineered molecules resulting from modifications

of glycans or of amino acid composition, or from conjugation to other molecules, such as in the case of pegylated proteins. These alterations in the protein are carried out with different goals: (i) to improve the pharmacokinetic profile of the protein; (ii) to increase protein half-life; and/or (iii) to alter its immunogenic profile.

An example of a second-generation biopharmaceutical obtained by site-directed mutagenesis is TNKase (Tenecteplase™), which is an altered form of recombinant tPA produced in CHO cells by Genentech. The substitution of three amino acids in the native protein results in a molecule with higher affinity for its receptor (fibrin), a higher resistance to its natural inhibitor (PAI-1), and an increased half-life (Walsh, 2004).

Second-generation insulins are also available and consist of molecules with changes in one to two amino acids, aimed at accelerating the onset of activity (Lispro and Aspart insulin products) or increasing the half-life (Glargine insulin product).

Humanized antibodies, discussed in more detail in Chapter 17, are also considered second-generation molecules. By humanization, the high immunogenicity of a murine antibody is eliminated, enabling improved therapeutic use.

Protein engineering can also be used for creating fusion proteins or hybrid biopharmaceuticals. An example of this type of product is Enbrel® (Etanercept), which consists of the extracellular ligand-binding domain of the 75-kDa receptor for tumor necrosis factor-alpha (TNFα) and the Fc portion of human IgG1. TNF is a proinflammatory cytokine present at high concentrations in patients suffering from rheumatoid arthritis. Enbrel® acts as competitive inhibitor of TNF, as it competes with the receptor molecules (TNFR) present in the cell. The antibody portion of the fusion protein increases the half-life in blood (Walsh, 2004).

Another approach relies on modifying the glycan of glycoproteins to improve pharmacokinetics of biopharmaceuticals. An example was the introduction of two additional N-glycosylation sites in EPO, resulting in a hyperglycosylated erythropoietin product (Aranesp®), which was approved for commercialization in 2001. The modification of the carbohydrate structure increased the half-life to 25.3 hours (as compared with 8.5 hours for the first-generation EPO), allowing a decrease in injection frequency that is attractive for patients.

With the considerable evolution achieved in recent years in the elucidation of structure–function relationship of proteins, as well as in the areas of protein engineering and bioinformatics, many new developments are expected in the field of new-generation biopharmaceuticals. These will have significant advantages for patients, including lower immunogenicity, lower frequency of injections, and enhanced stability in serum.

References

Anderson PM, Markovic SN, Sloan JA, Clawson ML, Wylam M, Arndt CAS, Smithson WA, Burch P, Gornet M, Rahman E (1999), Aerosol granulocyte macrophage colony stimulating factor: a low toxicity, lung specific biological therapy in patients with lung metastases, Clin. Cancer Res. 5:2316 2323.

Baneyx F, Mujacic M (2004), Recombinant protein folding and misfolding in *Escher ichia coli*, Nat. Biotechnol. 22:1399 1408.
Bhattacharya SK (2004), The use of enzyme mixtures for complex biosyntheses, Curr. Opin. Biotechnol. 15:449 455.
Bhattacharyya MS, Singh J, Soni P, Banerjee UC (2003), Recombinant factor VIII for haemophilia: an overview of production technologies, CRIPS 4:2 8.
Boedeker BGD (1992), The manufacturing of the recombinant factor VIII Kogenate®, Transf. Med. Rev. 6:256 260.
Castilho LR, Medronho RA (2002), Cell retention devices for suspended cell perfusion cultures, Adv. Biochem. Eng. Biotechnol. 74:129 169.
Chiba Y, Suzuki M, Yoshida S, Yoshida A, Ikenaga H, Takeuchi M, Jigami Y, Ichischimak K (1998), Production of human compatible high mannose type ($Man_5GlcNAc_2$) sugar chains in *Saccharomyces cerevisiae*, J. Biol. Chem. 273:26298 26304.
Chirino AJ, Mire Sluis A (2004), Characterizing biological products and assessing comparability following manufacturing changes, Nat. Biotechnol. 22:1383 1391.
Crommelin DJA, Storm G, Verrijk R, Leede L, Jiskoot W, Hennink WE (2003), Shifting paradigms: biopharmaceuticals versus low molecular weight drugs, Int. J. Pharm. 266:3 16.
Dudzinski DM (2004), Reflections on historical, scientific, and legal issues relevant to designing approval pathways for generic versions of recombinant protein based therapeutics and monoclonal antibodies, Harvard Law School, Thesis. Available at: http://leda.law.harvard.edu/leda/data/630/(accessed February 2006).
Edwards J, Kirby N (1999), Recombinant coagulation factor IX (BeneFix®). In: Walsh G, Murphy B (Eds), Biopharmaceuticals: An Industrial Perspective, Springer Verlag, Berlin, pp. 73 108.
European Medicines Agency (2005), Guidance documents on biosimilar products. Available at: http://www.emea.eu.int/htms/human/biosimilar/biosimilarfin.htm (accessed October 2005).
European Medicines Agency (2006), EPARs for authorised medicinal products for human use. Available at: http://www.emea.europa.eu/htms/human/epar/a.htm (accessed December 2006).
Food and Drug Administration (2005), Scientific considerations related to developing follow on protein products. Available at: http://www.fda.gov/cder/meeting/followOn/followOnPresentations2 2005.htm and http://www.fda.gov/cder/meeting/followOn/followOnPresentations.htm (accessed May 2005).
Food and Drug Administration (2006), FDA approved products. Available at: http://www.accessdata.fda.gov/scripts/cder/drugsatfda/(accessed December 2006).
Gerngross TU (2004), Advances in the production of human therapeutic proteins in yeast and filamentous fungi, Nat. Biotechnol. 22:1409 1414.
Hellwig S, Drossard J, Twyman RM, Fischer R (2004), Plant cell cultures for the production of recombinant proteins, Nat. Biotechnol. 22:1415 1422.
Katsnelson A, Ransom J, Vermij P, Waltz E (2006), News in brief: USDA approves the first plant based vaccine, Nat. Biotechnol. 24:233 234.
Khmelnitsky YL (2004), Current strategies for in vitro protein glycosylation, J. Mol. Catal. B Enzym. 31:73 81.
Kwon TH, Kim YS, Lee JH, Yang MS (2003), Production and secretion of biologically active human granulocyte macrophage colony stimulation factor in transgenic tomato suspension cultures, Biotechnol. Lett. 25:1571 1574.
Lee SY, Kim DI (2002), Stimulation of murine granulocyte macrophage colony stimulation factor production by pluronic F 68 and polyethylene glycol in transgenic *Nicotiana tabacum* cell culture, Biotechnol. Lett. 24:1779 1783.
Martinet W, Maras M, Saelens X, Jou WM, Contreras R (1998), Modification of the

protein glycosylation pathway in the methylotrophic yeast *Pichia pastoris*, Bio technol. Lett. 20:1171 1177.
Muller RH, Keck CM (2004), Challenges and solutions for the delivery of biotech drugs a review of drug nanocrystal technology and lipid nanoparticles, J. Biotechnol. 113:151 170.
Parker MH, Birck Wilson E, Allard G, Masiello N, Day M, Murphy KP, Paragas V, Silver S, Moody MD (2004), Purification and characterization of a recombinant version of human α fetoprotein expressed in the milk of transgenic milk, Prot. Express. Purif. 38:177 183.
Pavlou AK, Belsey MJ (2005), The therapeutic antibodies market to 2008, Eur. J. Pharm. Biopharm. 59:389 396.
PhRMA (2004), 324 Biotechnology medicines in testing promise to bolster the arsenal against disease. Available at: http://www.phrma.org/files/Biotech%20Survey.pdf (accessed February 2006).
Reichert J, Pavlou A (2004), Monoclonal antibodies market, Nat. Rev. Drug Discov. 3:383 384.
Research and Markets (2005a), Biopharmaceuticals current market dynamics & future outlook. Available at: http://www.researchandmarkets.com/reports/39083 (accessed May 2005).
Research and Markets (2005b), Erythropoietin current market dynamics & future outlook. Available at: http://www.researchandmarkets.com/reportinfo.asp?report id=298258 (accessed May 2005).
Research and Markets (2005c), Colony stimulating factors current market dynamics & future outlook. Available at: http://www.researchandmarkets.com/reportinfo.asp?report id=235022 (accessed May 2005).
Research and Markets (2005d), Monoclonal antibody therapeutics: current market dynamics & future outlook. Available at: http://www.researchandmarkets.com/reportinfo.asp?report id=75046 (accessed May 2005).
Ryff JC, Schellekens H (2002), Immunogenicity of rDNA derived pharmaceuticals, Trends Pharmacol. Sci. 23:254 256.
Walsh G (2002), Biopharmaceuticals and biotechnology medicines: an issue of nomen clature, Eur. J. Pharm. Sci. 15:135 138.
Walsh G (2003), Biopharmaceuticals Biochemistry and Biotechnology, 2nd Ed., John Wiley and Sons, Chichester.
Walsh G (2004), Second generation biopharmaceuticals, Eur. J. Pharm. Biopharm. 58:185 196.
Werner RG (2004), Economic aspects of commercial manufacture of biopharmaceuti cals, J. Biotechnol. 113:171 182.

Monoclonal antibodies

17

Wirla M.S.C. Tamashiro and Elisabeth F.P. Augusto

17.1 Introduction

Antibodies are molecules secreted by plasmocytes; these are differentiated from B lymphocytes following previous contact with a specific antigen. The number of different antibodies produced by humans is extremely large, incorporating between 10^7 and 10^8 distinct molecules, but any one B lymphocyte only produces antibodies against a single specific antigen. After immunization or contact with the antigen, the B cells which recognize each antigenic determinant in an antigen molecule will produce clones, thus increasing the number of lymphocytes specific for that antigen. Although the antibodies produced by all of these lymphocyte clones will be specific for the same antigen, these antibodies will not necessarily be structurally identical, that is, they are polyclonal antibodies. The result of this process is an increase in the level of specific antibodies in the serum or body secretions of the immunized individual. Even immunization with highly purified antigens results in the production of polyclonal antibodies, so that the composition and properties of immune sera vary with each preparation (Abbas *et al.*, 2000).

Attempts to produce homogeneous antibodies *in vitro* arose almost as soon as lymphocytes and their biological properties were discovered. However, B lymphocytes normally do not survive in cell cultures. For survival, they must undergo a malign transformation, such as that induced by the Epstein–Barr virus (Steinitz *et al.*, 1977). Some tumors of B lymphocytes, the multiple myelomas, can arise spontaneously and others can be induced by the administration of mineral oil. These tumors, whether spontaneous or induced, can be adapted to *in vitro* cultivation, and they will secrete highly homogeneous antibodies (monoclonal antibodies, mAbs) since they originate from a single tumor cell (Horibata and Harris, 1977). However, even if all antibodies are homogeneous in a myeloma culture, it is impossible to predict what this specificity will be.

The production *in vitro* of mAbs with a predetermined specificity has only been possible since the advent of the technology of hybridomas, which was introduced by Kohler and Milstein in 1975. These hybridomas are the products of *in vitro* fusion of myelomas with normal B lymphocytes. The fusion products preserve the capacity for self-propagation in a culture, as well as the secretion of the antibodies of interest, characteristics inherited from the parent myeloma and the normal B lymphocyte, respectively. The myelomas used in such fusions generally involve cell lines from B-lymphocyte tumors developed in mice or rats (Cotton and Milstein, 1973; Köhler and Milstein, 1975b; Shulman *et al.*, 1978), while

the B lymphocytes come from mice or rats previously immunized with the antigen of interest. After the fusion of the myeloma with the normal B lymphocyte, the hybridomas of interest can be selected and cloned to obtain an unlimited quantity of homogeneous antibodies that are highly specific (Köhler and Milstein, 1975a).

The production of such mAbs using hybridoma technology has made it possible to detect and quantify a wide variety of molecules produced by live organisms in highly specific and sensitive assays, thus making possible enormous advances in various areas of biological research. The specificity of these mAbs has also facilitated the improvement of clinical diagnoses, as well as creating expectations about their use as a therapeutic agent for human diseases.

One problem with the use of these antibodies in humans, however, is the immunogenicity (the ability to induce an immune response) of murine mAbs, and various associated adverse reactions. Some mouse mAbs have, however, been licensed for use in human patients (Lin *et al.*, 2005). The adverse reactions can be controlled to a certain extent by ''humanization'' of the murine reagents (produced by cells transfected with antibody genes), as well as by the use of recombinant DNA techniques to produce antibody fragments preserving the antigen-binding capacity of the original antibody molecules (Huhalov and Chester, 2004). Only recently has the establishment of a line of human myeloma cells (called Karpas 707H) been reported, and this is proving useful in the production of hybridomas via fusion with B lymphocytes obtained from immunized or infected humans (Karpas *et al.*, 2001; Vaisbourd *et al.*, 2001). Obtaining all these highly specific mAbs, whether murine, humanized, or antibody fragments, is dependent upon the production of a hybridoma.

Both hybridomas and humanized antibody producer cells can be cultivated indefinitely in conventional cultures, usually containing fetal bovine serum in the medium. In the supernatants of these cultures, the quantity of mAbs varies from 20 to 100 μg/ml of protein, depending on the cell and the system of cultivation.

There are, however, *in vivo* methods of obtaining mAbs, and these produce much larger quantities. One of these is intraperitoneal administration of the hybridomas into histocompatible animals (of the same cell line as the parents of the hybridoma) or immunodeficient animals (individuals with no functional immune system). These receptor animals will develop ascitic tumors containing from 1 to 40 mg/ml of the mAb secreted by the hybridoma (Kretzmer, 2002). However, despite the fact that this method is well documented and has been used widely in the past, the procedure is presently being rejected because of ethical concerns over the use of laboratory animals.

This has led to an increased interest in methods for the *in vitro* production of large quantities of mAbs. Submersed cultivation in bioreactors is one possibility. In contrast with traditional methods, this procedure uses large quantities of culture medium, increasing the scale of the process and making possible the production of virtually unlimited quantities of mAbs by a given hybridoma.

In this chapter, the fundamentals of hybridoma technology are discussed and the most widely used protocols and procedures are presented.

The main advances in large-scale *in vitro* production of mAbs are also presented. This topic is of extreme importance in the biotechnology industry, as can be seen by the extent of the use of mAbs in diagnostic and therapeutic applications.

17.2 Antibodies

Antibodies constitute the prototype of the superfamily of immunoglobins (Ig), molecules, which have globular domains resulting from the existence of cysteine residues along their polypeptide chains. These residues form disulfide bridges (S-S) within and between chains. The functional units of the antibody molecules are generally monomers, each formed by a pair of light polypeptide chains of approximately 25 kDa each, and another pair of heavy ones of about 50 kDa (*Figure 17.1*). The chains of each pair are identical, with all four chains linked by inter-chain disulfide bridges. Each of the antibody chains in this monomer has an N-terminal domain, identical for the two members of each pair, although these domains vary from one molecule to another. The heavy chains also have three or more constant domains (C-terminals), whereas the light chains have only a single one. The variable N-terminal domains of the Ig molecule constitute the antigen combining sites, with each of the two sets of heavy and light chains of the monomer constituting a single site for antigen binding. Hence, each monomer of the immunoglobulin has duplicate sites for combining with a specific antigen. The rest of the molecule (the C-domains) effects the other functions of the antibody: interaction with the cells of the immune system (via Ig receptors), linking to the molecules of the complement system, and crossing the placenta or epithe-

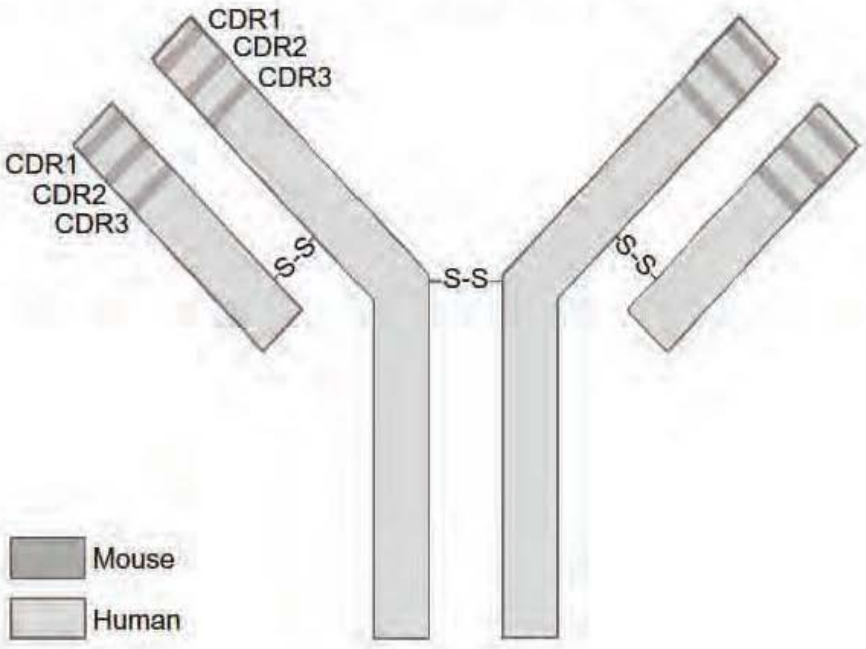


Figure 17.1

Structure of an antibody molecule.

lial mucosa. The Ig classes (IgD, IgM, IgG, IgE, and IgA) are determined by the primary sequence of the constant domains of the heavy chain pair. IgD and IgM monomers are expressed on the surface of the naïve B lymphocyte, which constitute B-cell receptors (BCRs) for antigens. Except for IgD, all of the Ig classes can be secreted by plasmocytes upon contact with a specific antigen, whether a foreign substance or an altered native constituent. Although IgG and IgE are always monomers, IgA molecules can be secreted as either monomers or dimers. IgM is always secreted as a pentamer. In humans, there are four IgG subclasses: IgG1, IgG2, IgG3, and IgG4, whereas there are two IgA subclasses: IgA1 and IgA2 (Abbas *et al.*, 2000).

There are, however, enormous numbers of antigens that may be encountered by the immune system. To deal with them, B lymphocytes undergo a unique developmental process involving genetic recombination of gene segments to form a wide variety of antibodies. This process, which takes place in the bone marrow of mammals and the Fabricius bursa of birds, was determined some 30 years ago by Tonegawa *et al*. (1978). The critical aspect of this process is the occurrence of cuts in the parent DNA chain of the developing lymphocyte by special enzymes (RAG1 and RAG2), that are only found in these cells. This cutting and the subsequent rearrangement of gene segments allows great variety in the gene, which is responsible for the formation of the N-terminal (variable) regions of the Ig chains. This enables coupling with the wide variety of antigen determinants encountered by the organism.

Three regions of the parent DNA heavy chain are affected by these cuts: V, D, and J (for variable, diversity, and junction, respectively). Initially, simultaneous cuts occur in a D and a J region of the chain; the segment between the two cuts is eliminated, and the two loose ends are joined in a DJ sequence. Then, two additional cuts occur, one in a V region, and the second immediately prior to the new DJ sequence. The intervening segment is eliminated, and the V region is attached to the DJ sequence to form the final VDJ gene sequence, which codifies the variable portion of what will be the heavy Ig chain. *Figure 17.2* shows this gene recombination in the formation of the heavy chain variable region.

The second step in the formation of the heavy chain involves transcription of the DNA to form the primary transcript of the RNA of the developing lymphocyte, which includes the new VDJ sequence. This primary transcript (tRNA), however, still includes non-codifying segments (introns). In the third step, these introns are eliminated, and the final RNA includes only the variable N-terminal gene and the genes for all of the constant regions.

This mRNA is then translated to heavy polypeptide chains in the cytoplasm. The first set formed is the heavy chain of IgM, consisting of a VDJ region and a Cμ region (the first of the sequence of constant genes) (Maki *et al.*, 1980). Each pair of these heavy chains is joined with a pair of surrogate light chains (formed without genetic recombination) and migrates to the surface of the developing lymphocyte. This provisional antibody probably attaches to a self-antigen, triggering the rearrangement of the genes for the variable region of the light chains. This rearrangement is similar to that undergone in the formation of the heavy chain, except

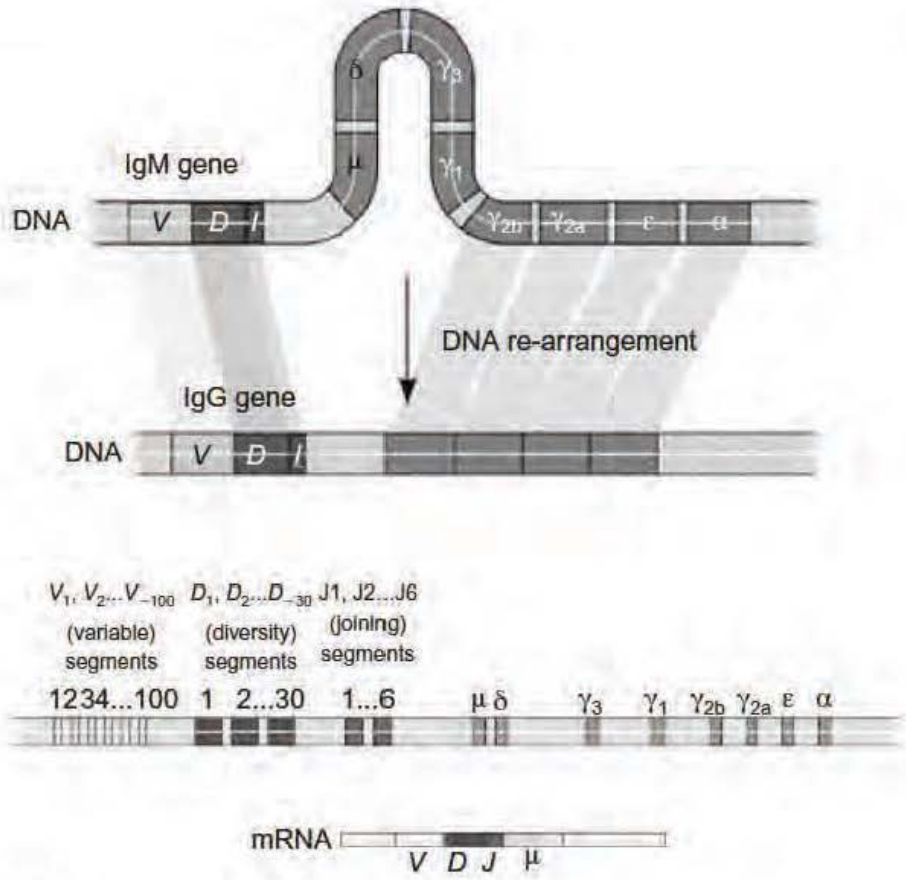


Figure 17.2

Genes for the expression of immunoglobulins heavy chain.

that only a single pair of cuts in the V and J regions is involved, because there is no D region in light chains.

The first or primary transcript of this light chain gene segment includes both the VJ gene sequence and the two genes for the constant region (kappa and lambda). The formation of the mRNA for the light chains is similar to that of the heavy chains. Kappa chains are the first light chains to be formed and lambda chains will only be formed if the molecules are non-functional.

Once the definitive light chains have been produced, they replace the surrogate chains in the constitution of the antigen receptor of developing B lymphocyte, and then the immature lymphocyte migrates to the peripheral lymphoid organs (spleen, lymph nodes, etc.). Once definitive chains have been produced, their presence inhibits rearrangement of the genes of the corresponding allele. This process, called allele exclusion, leads to the production of surface immunoglobulins capable of recognizing only one specific antigenic determinant. The set of genetic events described here is responsible for the generation of the enormous repertoire of antibodies in organisms with an immune system (Sakano *et al.*, 1980).

During the mid-1970s, this process of antibody generation was not understood, and the laboratory of Cesar Milstein was one of those trying to decipher the mechanisms involved. The experimental approach adopted

by this group involved the fusion of myelomas from mice with those from rats using the Sendai virus. The resultant hybrid cells were found to be able to express complete molecules from both of the parent cells, as well as mixed proteins formed by the light chains of one parent and the heavy chains of the other. Given these results, he corroborated the hypothesis of allele exclusion of Ig genes, which had been proposed several years earlier (Pernis *et al.*, 1965), but he also showed that the joining of light and heavy chains was not a selective process, that is, any light or heavy chain available in the cytoplasm of the hybrid cell could be included.

Once inter-species fusion was shown to be possible, the next step was an attempt to fuse myelomas with normal B lymphocytes. Only in 1975 did Kohler and Milstein propose a protocol that led to the efficient production of hybrid cells for the secretion of mAbs with a predetermined specificity, which could be perpetuated in cell cultures, the so-called hybridomas (Köhler, 1981).

For the isolation of these hybridomas to be feasible, the myeloma line must be deficient in one of the two paths of DNA synthesis. The myeloma cells used by Kohler and Milstein for the production of the first hybridomas were deficient in hypoxanthine phosphoribosyl transferase (HGPRT), an enzyme involved in the synthesis of DNA nucleotides. When these myelomas were put into contact with the spleen cells of mice previously immunized with sheep erythrocytes, and an inactivated Sendai virus added as a fusion agent, the resulting fusion products included hybridomas producing the mAb of interest. By removing the fusion agent and seeding the cells in the presence of aminopterin, an inhibitor of the salvage pathway of DNA synthesis, they were able to isolate the effective hybridoma, because growth of the myeloma cells not fused with the spleen cells is completely inhibited.

Approximately a week after plating, all the normal (non-fused) B lymphocytes had already been eliminated naturally from the culture, because normal lymphocytes do not propagate in culture. Only those hybrids resulting from the fusion of the myelomas with the B lymphocytes were able to grow in the culture, and some of them also preserved the ability to secrete antibodies against the antigen used for immunization.

Today the myelomas most frequently used in the production of hybridomas are still those that have a mutation in the gene that codifies the HGPRT enzyme (Littlefield, 1964), and show growth inhibition in a medium containing 8-azaguanine or 6-thioguanine, both analogs of guanine. Other mutant cell lines lacking the enzyme thymidine kinase (TK) also exist, but are less frequently used. Their hybridomas can be identified due to their ability to survive in a medium containing 5-bromodeoxiuridine, an analog of thymidine.

HGPRT and TK enzymes are important in the synthesis of DNA from preformed nucleotides provided in the culture medium. The myeloma cells lacking these enzymes are unable to utilize the hypoxanthine or thymidine from the medium; moreover, they die in the presence of aminopterin, which inhibits the *de novo* synthesis of DNA. Thus, in a medium containing aminopterin, hypoxanthine, and thymidine (HAT medium) only those hybridomas that receive the HGPRT or TK enzyme from the normal parents (spleen B lymphocytes) will survive.

The standard protocol used today for the production of hybridomas is somewhat different from that employed by Kohler and Milstein in 1975. The major modifications (proposed by De St Groth and Scheideger (1980)) involve the use of polyethylene glycol (PEG) as a fusion agent, the use of myeloma cells that do not produce antibodies, the cloning of hybridomas using limitant dilution, and cultivation on plastic plates.

17.3 Production of monoclonal antibodies

Five steps are important in the production of hybridomas to be used for the secretion of mAbs of a determined specificity (Nelson *et al.*, 2000): (1) immunization of mice, (2) fusion and selection of secreting hybridomas; (3) cloning of hybridomas, (4) definition of the isotype of mAbs obtained, and (5) further development (*Figure 17.3*).

17.3.1 Step 1: Immunization

Most myeloma lines used in cell fusion originate from BALB/c mice. These mice can be immunized with exogenous proteins (50–100 μg/ml), with cells (10^7 cells) or with peptides conjugated with carrier proteins, such as the keyhole limpet hemocyanin (KLH). The proteins and peptides

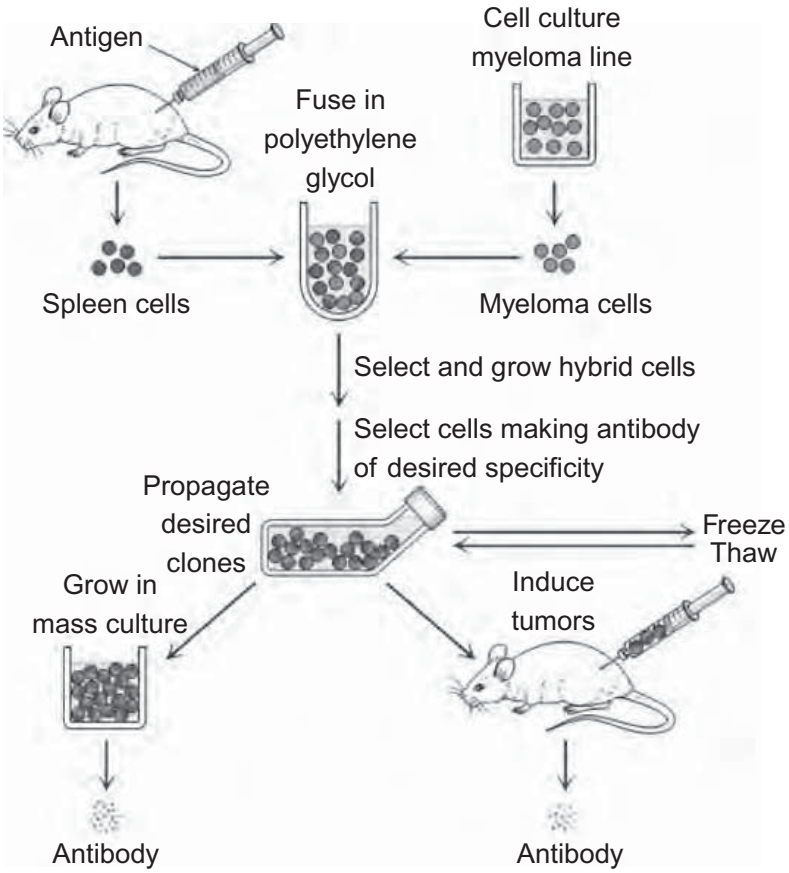


Figure 17.3

Scheme for hybridoma production.

are generally mixed with adjuvants (complete or incomplete Freund adjuvant, potassium alum ($K_2Al_24SO_4 + 24H_2O$) or commercial adjuvants such as Titer Max) and introduced subcutaneously or intraperitoneally. Cells are generally administered intraperitoneally in the absence of adjuvants. It is usually necessary to repeat the administration of the antigen once or twice to obtain an immune response with a high level of antibodies. The level of specific antibodies in the serum is assessed by immunoenzymatic assays of the ELISA type, using serum separated from a small sample of blood collected from the tail or the ocular plexus veins.

The immunization of laboratory animals such as mice or rats is designed to increase the number of B-lymphocyte clones specific for the antigen, thus increasing the chances of obtaining hybridomas that will secrete the antibodies of interest in fusion experiments. The booster doses promote the switch of immunoglobulin class and the maturation of antibody affinity due to somatic hypermutation of the variable genes for the immunoglobulins that arise after repeated exposure of the animal to the antigen.

17.3.2 Step 2: Fusion and selection of secreting hybridomas

Myeloma cells are generally of the SP2Ag14/0 line (Köhler and Shulman, 1978); they are cultivated in RPMI 1640 medium containing 10% fetal calf serum until semiconfluence and then collected from the culture flasks by centrifugation.

Animal spleens that present the highest antibody levels are collected aseptically, disrupted in the culture medium, and then the spleen cell suspensions are transferred to the centrifuge tube containing the myelomas. The mixture contains 2×10^7 myeloma cells for each 10^8 spleen cells. These cells are allowed to sediment and are then washed twice with a serum medium and centrifuged.

The cell mixture is then resuspended in 1 ml of a 10% DMSO and 50% PEG solution. This solution is added slowly to the cells over a period of 2.5 minutes. The first 60 seconds are at room temperature, after which the temperature is increased to 37°C (for the final 90 seconds). The volume of the cell suspension is then slowly increased to a total of 50 ml with culture medium or physiological saline solution. After 5 minutes the cells are allowed to sediment and washed twice by centrifugation. The final sediment is resuspended in HAT medium containing 20% fetal bovine serum. In the standard procedure, the cells are now plated at a density of 10^5 per well in 96-well plates containing a feeder layer of macrophages, although in some protocols, subsequent selection is simplified by plating 24 wells with a density of 10^6 cells per well. The feeder layer is prepared by seeding the wells with macrophages collected from the peritoneal cavity of normal mice some 48 hours prior to the fusion procedure. In addition to providing growth and differentiation factors, such as interleukin (IL)-6, this feeder layer provides the cell density necessary for the growth of the hybridomas that manage to survive the process.

Some 10–15 days after seeding, the hybridomas are already sufficiently established on the surface of the plate that the antibodies of interest can be detected in the supernatants of the cultures. Their identification involves

the use of specific assays, which are defined by the nature of the antigen and the properties of the antibody being produced.

For cell surface antigens, the most frequently used assays are immunofluorescence or immunoenzymatic, with their numerous variations. For soluble protein or peptide antigens, the most common assays are immunoenzymatic, enzyme-linked immunosorbent assays (ELISA) or Western blot tests.

17.3.3 Step 3: Hybridoma cloning

Once the antibody-secreting hybridomas are obtained, they are cloned by limiting dilution, a procedure which consists of seeding a 96-well plate with equal aliquots of a 100-cell suspension of the clones, which will hypothetically result in cultures of one cell per well. Some 10 days after the cloning, the culture plate is investigated to determine whether or not clones are actually growing. These clones are similar in appearance to a bacterial colony in culture. The wells are then tested for the presence of the antibody, and each clone with the proper characteristics is expanded in culture flasks. The culture will now secrete a single type of antibody, known as an mAb. Once hybridoma clones are established, they can be propagated *in vitro* indefinitely. An alternative means of propagation involves *in vivo* intraperitoneal inoculation in histocompatible animals previously inoculated with mineral oil (Pristane or Nujol), since these animals will develop ascitic tumors. These tumors accumulate a liquid that will contain large quantities of the mAbs. The hybridomas can also be frozen in liquid nitrogen for future use, thus providing an unlimited source of specific antibodies.

17.3.4 Step 4: Definition of the isotype of monoclonal antibodies obtained

Depending on the isotype (i.e. class/subclass and kind of light chain), immunoglobulin molecules will display a particular biological property and will require an appropriate method for purification. The identification of mAb isotypes generally employs the culture supernatants of hybridomas and commercially available kits for the specific immunoenzymatic assays. This knowledge about the specific isotype facilitates the selection of the purification process in the next step.

17.3.5 Step 5: Follow-up/later developments

Depending on the use of the mAbs, certain adaptations may be required for their preparation. When large quantities of mAbs are required, *in vivo* production of the antibody in ascitic fluid is not practical, because it will require the use of a large number of animals. Thus, it is often easier to cultivate the antibodies in an appropriate *in vitro* culture medium. However, given the strict nutritional requirements of the hybridomas and their fragility in the face of osmolality, pH variations, and the accumulation of metabolites, the production of large quantities of antibodies *in vitro* will necessitate special care.

The purification of mAbs of the IgG class is usually carried out by affinity chromatography on a resin coated with protein A or protein G from *Staphylococcus* sp., ligands that have affinity for this kind of immunoglobulin. More recently, other affinity ligands have been introduced, such as protein L, which has a special affinity for kappa light chains. Other Ig molecule classes, especially those with lambda light chains, can be purified by other methods, such as pseudo-bioaffinity. In this case, the chromatography uses metallic ions or certain dyes as ligands. These will interact with the antibody molecules due either to electric charge or to hydrophobic reactions (see Chapter 12 and review by Vijayalakshmi, 1989).

After purification, mAbs can be covalently linked with other reagents for use in specific assays. These reagents include radioisotopes (for radioimmunoassays or the *in vivo* tagging of antigens), enzymes (for immunoenzymatic assays), or fluorochromes (for immunofluorescence assays). All of the procedures require time and exhaustive work in reagent standardization, but the most complex are those produced for therapeutic use in humans. Some of the developments in obtaining reagents for human applications will be discussed here, especially the use of recombinant DNA technology.

17.4 Production of recombinant antibodies

The development of the technique for the construction of hybridomas has made possible the rapid dissemination of mAbs as an analytic tool, and these products have had a profound impact on the procedures of diagnosis and the purification of other proteins. Although numerous applications of mAbs have been developed, the greatest interest has always been in medicinal uses. However, the mAbs obtained from hybridomas have – at least initially – proved to be less efficient as therapeutic agents. Problems include insufficient activation of effector functions in humans and the stimulation of an immune response to the rodent proteins. This latter phenomenon, known as the HAMA response (for human anti-murine antibody), results from the fact that murine antibodies are recognized as antigens by the human immunological system, and they will be rapidly eliminated from circulation by antimurine antibodies, thus reducing the effects of the treatment. To overcome this difficulty, larger doses of the medicine are required, although this will increase the risk of undesirable effects.

These problems explain why so few products of murine origin have been launched on the market (*Table 17.1*). The only therapeutic product of murine origin that is well established is OKT3® mAb. This antibody is used in the reversal of acute transplant rejection. It involves an anti-CD3 (cluster of differentiation 3) antibody, which is part of the receptor of the membrane of T lymphocytes, enabling the temporary elimination of the T lymphocytes involved in the rejection process from circulation, thus facilitating the acceptance of the transplanted organ. Since the treatment required is of short duration, the immune response of the patient against the murine antigen, if it occurs, can be kept to acceptable levels.

Table 17.1 Approved human monoclonal antibodies

Company	Commercial names (generic names)	Type	Category	Approval date
Johnson & Johnson	Orthoclone® OKT3® (Muromonab CD3)	Murine	Immunologic*	1986
Centocor	ReoPro® (Abciximab)	Chimeric	Heart ischemic disease	1994
Centocor/Glaxo	Panorex® (Edrecolomab)	Murine	Antineoplastic	1995
Biogen IDEC	Rituxan® (Rituximab)	Chimeric	Antineoplastic	1997
Hoffmann La Roche	Zenapax® (Daclizumab)	Humanized	Immunologic*	1997
Novartis	Simulect® (Basiliximab)	Chimeric	Immunologic*	1998
MedImmune	Synagis® (Palivizumab)	Humanized	Anti infection	1998
Centocor	Remicade® (Ifiximab)	Chimeric	Immunologic*	1998
Genentech	Herceptin® (Trastuzumab)	Humanized	Antineoplasic	1998
Wyeth	Mylotarg® (Gemtuzumab ozogamicin)	Humanized	Antineoplastic	2000
Millenium/ILEX	Campath® (Alemtuzumab)	Humanized	Antineoplastic	2001
Biogen IDEC	Zevalin® (Ibritumomab tiuxetan)	Murine	Antineoplastic	2002
Abbott	Humira® (Adalimumab)	Human	Immunologic*	2002
Genentech	Xolair® (Omalizumab)	Humanized	Immunologic*	2003
Corixa	Bexxar® (Tositumomab I131)	Murine	Antineoplastic	2003
Genentech	Raptiva® (Efalizumab)	Humanized	Immunologic*	2003
Imclone System	Erbitux® (Cetumimab)	Chimeric	Antineoplastic	2004
Genentech	Avastin® (Bevacizumab)	Humanized	Antineoplastic	2004

Adapted from Reichert and Pavlou (2004).
*Including arthritis, immunological and inflammatory diseases, and prevention of transplantation rejection.

Ideally, mAbs for therapeutic use should be completely human (van Dijk and van de Winkel, 2001; Roque *et al.*, 2004). However, the construction of hybridomas capable of producing such proteins presents both technical and ethical problems. The immunization of humans is unacceptable, especially since this would entail a biopsy for the collection of secondary lymphoid organs. One alternative would be the *in vitro* production of human mAbs using B lymphocytes collected from the peripheral blood of naive or naturally immunized human donors. In both of these procedures, however, the frequency of B lymphocytes specific for a given antigen is quite low. Moreover, B lymphocytes producing antibodies against self-antigen, frequent targets of mAbs, are generally extremely rare, since such B lymphocytes will normally be eliminated before maturation by a process known as ''tolerance induction.'' If they could be isolated, these lymphocytes could be perpetuated by techniques such as immortalization by Epstein–Barr virus (EBV). However, the cells would be difficult to clone and have a low productivity of the antibody, as well as facing an inherent risk of contamination of the final product by viral particles (Little *et al.*, 2000).

Since it is impossible to obtain human mAbs from technologies such as those outlined above, other alternatives have been sought to enable the production of molecules with the appropriate variable domains of heavy and light chains, as well as its correct alignment, to guarantee the same affinity and specificity of the murine antibody, but with the human

characteristics of the constant regions. Various strategies have been tried, including recombinant DNA technology for the expression of mAbs on phages or in transgenic animals (Roque *et al.*, 2004). A result of the success of these strategies is the ever growing number of drugs for human use that have recently appeared on the market (*Table 17.1*). Moreover, some 2609 new products can be found in various phases of clinical trials (Reichert and Pavlou, 2004), and some 16 are expected to enter the market by 2008. It seems as if the potential of mAbs as therapeutic drugs has finally been realized.

Various procedures are used for obtaining humanized or human antibodies. Some of these using microbial or eukaryotic systems of cell expression will be presented further in this chapter. Other means of synthesis of these molecules, such as expression in plants, or even in the milk of transgenic animals, will not be discussed further.

17.4.1 Humanized antibodies

The technique of recombinant DNA provides a tool for the manipulation of the genes encoding antibodies present in murine hybridomas and those of human B lymphocytes that determine the expression of human antibodies, independent of specificity. It is thus possible to construct a genetic sequence that will express a molecule containing both the variable fragments (Fv) of the light and heavy murine chains of interest and the human functional fragments (Fc). Such a combination is called a chimeric antibody (*Figure 17.4*). These chimeric constructs can be inserted into cell lines, which will express antibodies that are 70% human, with half-lives and effector characteristics similar to those of the human molecule, although they preserve the specificity of the murine antibody. These chimeric antibodies still present a human antichimeric antibody (HACA) immune response, but this is less pronounced than that observed with the use of completely murine antibodies. Moreover, these humanized antibodies have been successful in numerous clinical treatments. There are currently five products on the market using this technology; they account for 70% of the sales of therapeutic antibody treatment. Moreover, a large number of chimeric antibodies can be found in various phases of clinical trials, and new ones are expected to be approved in the next few years (Reichert and Pavlou, 2004).

Humanized antibodies can also be made by genetic recombinant grafting so that specific regions of the chain that determine complementarity in the human molecule (complementarity-determining regions, CDRs) replace the murine ones, thus guaranteeing the specificity of the antibody produced by the hybridoma, but obtaining 90% of the properties of human antibodies. Each domain (VH and VL) has three of these CDR regions (*Figure 17.1*). These regions vary greatly and determine the specificity and affinity of binding sites of antibodies (Roque *et al.*, 2004). Like chimeric antibodies, these genetic constructs can be inserted into animal cells, which will then express the protein of interest in a suitable culture system.

Unlike the antibodies obtained by hybridoma technology, this type of antibody generally has a reduced affinity, but adverse reactions are

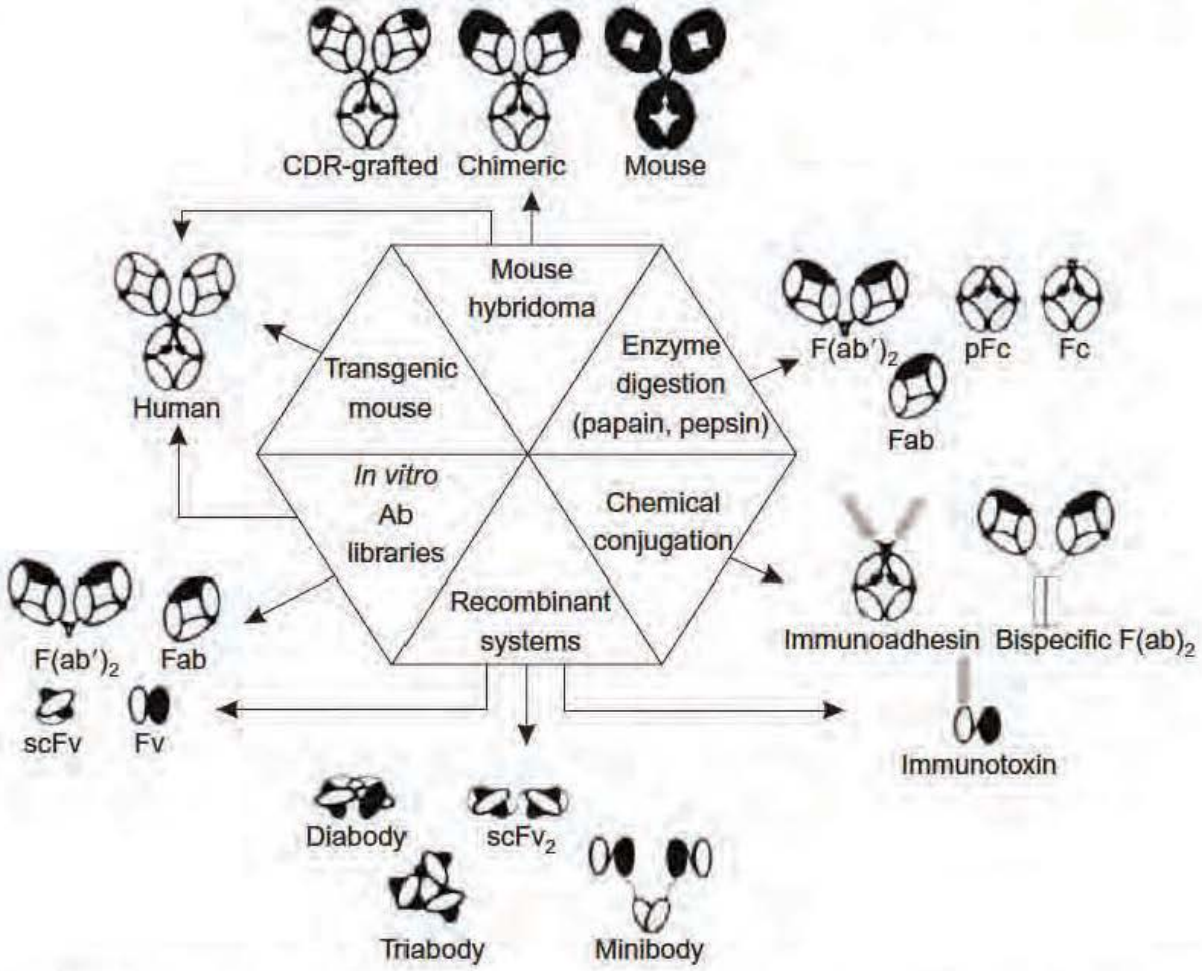


Figure 17.4

Recombinant antibodies: examples of possible types of constructions and technology employed (Reprint from Roque *et al.*, 2004. Copyright 2004 American Chemical Society).

of lesser intensity. At present, there are eight therapeutic products on the market that make use of this technology (*Table 17.1*), and the prospects for more are high, since some 42% of the mAbs in clinical trials involve molecules humanized by CDR grafting (Reichert and Pavlou, 2004).

17.4.2 Human antibodies

The same technologies used for obtaining humanized antibodies can be used for the production of fragments of completely human antibodies, which have tremendous advantages for clinical application. The most successful methods used to obtain human mAbs involve the construction of transgenic mice or the synthesis of human antibody fragments, based on "DNA libraries" of human cells, by using viral vectors to deliver the genetic material into cells inside a living organism or cultured *in vitro*.

Transgenic antibodies

In producing transgenic antibodies, it is the live mouse that is subjected to genetic modification, rather than the cells that will produce the humanized antibodies. Initially, the genes codifying the light and heavy chains are inactivated in an embryonic cell. Then large segments of DNA containing the genes for the light and heavy chains of human immunoglobulin are introduced into this cell. The cell will then grow into a transgenic mouse, which will be able to produce completely human antibody molecules. With this technology it should be possible to allow for isotype switching and affinity maturation. These transgenic mice can be immunized with any target molecule to obtain lymphocytes that synthesize human antibodies, with hybridomas being produced from these cells (van Dijk and van de Winkel, 2001; Roque *et al.*, 2004).

Another alternative is the insertion of small sequences of human chromosomes into embryonic animal cells, thus generating trans-chromosomic mice. These ''mini'' chromosomes are isolated from human chromosomes 2 and 14, which contain the genes for the light and heavy chains, respectively. This means that all of the V, D, and J segments of the variable N-terminal portion, as well as those of the constant regions, will become part of the mouse genome (van Dijk and van de Winkel, 2001; Roque *et al.*, 2004).

A third option uses ''trimera'' mice, which are mice that have been subjected to lethal irradiation, but are then prevented from suffering the effects of radiation by transplanting bone marrow cells from severe combined immunodeficient (SCID) mice, which have no B or T lymphocytes. To become trimera mice, the animals are repopulated with lymphocyte precursors from healthy human donors, and are then immunized with the antigen of interest. The immune system of the trimeras will then produce B lymphocytes that express specific human antibodies for the antigen, and their spleen cells can be used to produce hybridomas producing human immunoglobulins.

Antibody fragments

Various strategies have been used to combine the variable region of antibodies, which bind to the antigen determinants, with small functional proteins. Such constructs can be produced on a large scale in various expression systems (Irving *et al.*, 1996; Roque *et al.*, 2004); bacterial expression systems are relatively simple and less expensive than the alternatives, but eukaryote expression systems (yeast, mammalian, and insect cells) are also being used for this purpose (Roque *et al.*, 2004).

Most genetic constructs for obtaining antibody fragments express the Fv portions (*Figure 17.4*), which are the smallest antibody fragments that still retain the binding affinity of the parental antigen binding site (Irving *et al.*, 1996). These Fv fragments can also be expressed as single chain Fv molecules (scFv), or minibodies, in which variable domains of heavy and light chains are permanently linked by flexible peptide bridges (*Figure 17.4*) (Irving *et al.*, 1996; Roque *et al.*, 2004). This makes it possible to align the CDR regions of the chains in the same way that they were on the

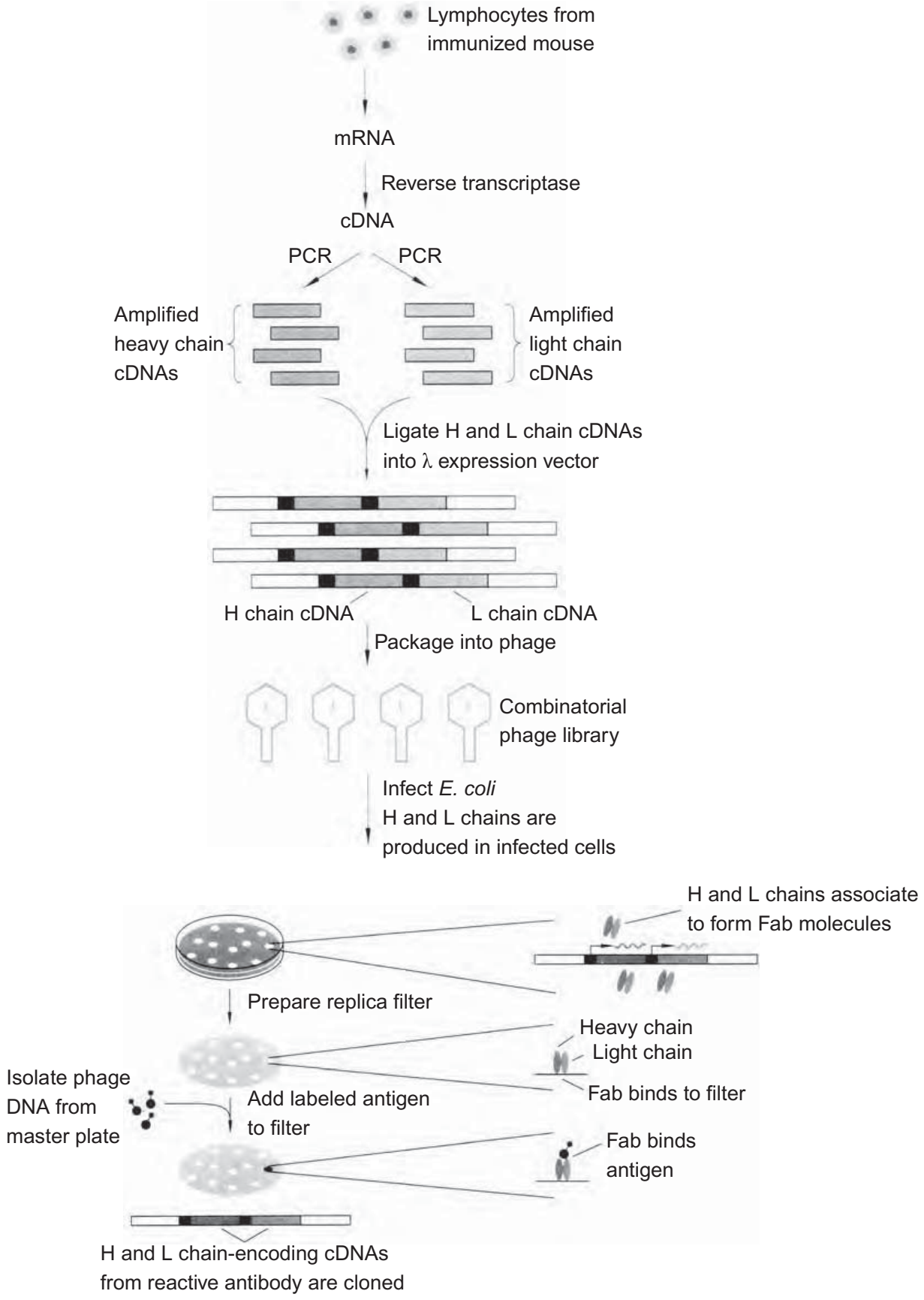
natural antibody (Irving *et al.*, 1996). The peptide bridges, with 10–25 amino acids, should preferentially be of a hydrophilic nature to prevent association with the hydrophobic V domains (I) and can incorporate tags that will be useful in the purification of the fragment after propagation in an expression system. One frequently used ligand is a combination of residues of glycine and serine (GLY4Ser)3 (Irving *et al.*, 1996).

The immunoglobulin genes can be obtained from either animals or humans, both naïve and immunized, although human sources are severely limited due to the ethical issues mentioned above. The repertoire of genes from immunized sources is smaller ($\sim 10^5$), but they normally generate antibodies of high affinity. These genes are usually stored in DNA libraries, which are cloned on phages. These phages serve as vectors for expression in eukaryotic cells, or in ribosomes, thus leading to the development of totally *in vitro* systems (van Dijk and van de Winkel, 2001; Roque *et al.*, 2004). The technique of phage presentation is widely utilized for the construction of these libraries (Emanuel *et al.*, 2000; Roque *et al.*, 2004). In this procedure, the Ab fragments are expressed as fusion proteins linked to the N-terminal of proteins on a viral surface, with various copies of the fusion proteins being expressed in the virus envelope (Irving *et al.*, 1996; van Dijk and van de Winkel, 2001).

Figure 17.5 illustrates the steps necessary for the construction of such a combinatory library of human DNA. Sequences of mRNA are isolated from lymphocytes of naive or immunized sources and utilized to synthesize the complementary DNA (cDNA), using the enzyme reverse transcriptase (Watson *et al.*, 1998). The polymerase chain reaction (PCR) then makes it possible to increase the number of gene sequences of both light and heavy chains of the immunoglobulin of interest. This is followed by linking these gene sequences to lambda vectors, thus creating two separate libraries (Emanuel *et al.*, 2000). These libraries can be combined by isolating the cDNA (previously stored on each of the phage types) coding the genetic sequence of the heavy and light chains. The two DNA segments are linked and packaged on a lambda expression vector so that each phage will contain a random pair of cDNA sequences, one for the heavy chain and the other for the light one.

These vector phages are used to infect a microorganism, usually *Escherichia coli*. The expression of the genes of interest can be monitored by the interaction of the expressed protein with the specific marked antigen, incorporated in the culture medium or in a cellulose-type filter. Once the vector expressing the genes that codify the fragment scFv have been identified, this can be used for cloning, usually as a plasmid in a microorganism. An alternative process is the use of the genes for the reconstruction of a complete chimeric antibody, as described above.

The stability and half-life of complete antibodies is greater than that of fragments. These characteristics are crucial in certain therapeutic applications (Hudson and Souriau, 2003). However, fragments are especially useful for diagnostic processes involving images, as well in the treatment of solid tumors, where good penetration of the tissues and rapid elimination from the bloodstream are desirable characteristics. They are also useful for inactivation of cytokines, neutralization of viruses, and blocking of receptors (Hudson and Souriau, 2003). The fragments which retain the


Lymphocytes from immunized mouse
mRNA
Reverse transcriptase
cDNA
PCR
PCR
Amplified heavy chain cDNAs
Amplified light chain cDNAs
Ligate H and L chain cDNAs into λ expression vector
H chain cDNA
L chain cDNA
Package into phage
Combinatorial phage library
Infect E. coli
H and L chains are produced in infected cells
H and L chains associate to form Fab molecules
Prepare replica filter
Heavy chain
Light chain
Isolate phage DNA from master plate
Add labeled antigen to filter
Fab binds to filter
Fab binds antigen
H and L chain-encoding cDNAs from reactive antibody are cloned

F(ab′)2 portion (*Figure 17.4*) normally last longer in circulation than do smaller fragments (Fab, Fv, or scFv). If necessary, however, the half-life of the smaller fragments can be increased by mixing them with a PEG, or by chemical conjugation to form dimers, trimers, and tetramers (''diabodies, ''triabodies'', and ''tetrabodies,'' respectively (*Figure 17.4*). These polymeric structures are obtained from the modulation of the length of the flexible peptide bridges of the scFv modules (Hudson and Souriau, 2003; Roque *et al.*, 2004).

It is also possible to produce bi-specific antibodies (*Figure 17.4*), which have two variable regions, each with a distinct specificity, but which bind, for example, to two adjacent epitopes of a given antigen, thus increasing the avidity of the connection of the antigen to the antibody. Bi-specific antibodies can also be generated so that they bind simultaneously to a tumor antigen and a cytotoxic T lymphocyte, thus facilitating the elimination of the tumor (Hudson and Souriau, 2003). These bi-specific antibodies can be produced by hybrid hybridomas (''quadromas''), by either chemical or genetic conjugation, as well as by the fusion of adhesive heterodimer domains of two or more Fab modules (Roque *et al.*, 2004).

Bi-functional antibodies can also be obtained from the fusion of natural antibodies or recombinant antibodies with compounds that might carry out auxiliary functions after antibody binding to its specific target (Hudson and Souriau, 2003; Roque *et al.*, 2004). These compounds can be radioactive conjugates, cytotoxic drugs, proteins, toxins, enzymes, and viruses, any of which can have diagnostic or therapeutic uses.

17.5 Production systems

The development of a specific process for the production of an mAb requires the selection of (a) a system of expression; (b) a bioprocess for obtaining the product, (c) a purification technique, and (d) analytical methods for determining purity and product quality. It is clear that all the choices must be compatible. So, for example, the selection of a process must be compatible with the expression system. Moreover, the process chosen must consider the specific conditions of the product in relation to the competitive market, the quality required, and the total volume to be produced.

Thus, for diagnostic or biogeneric industries, the production cost dominates the choices, and the optimization of the productive process is extremely important (NAS, 1999). This is not true for the therapeutic industry, or for products protected by patents, which are more dependent on the regulatory requirements.

Unlike the production of other proteins from animal cells, mAbs can also be produced *in vivo* by inducing ascitic tumors in laboratory animals,

Figure 17.5 (opposite)

Construction of a mAb combinatory library expressed in a bacterial system (from Recombinant DNA, 2/e by James D. Watson, *et al.* © 1992 by James D. Watson, Michael Gilman, Jan Wirkowski, and Mark Zoller. Used with permission).

as described above. This is an option when using hybridomas (Falkenberg, 1998; Hendriksen and Leeuw, 1998). However, there is a drive worldwide to find suitable *in vitro* production methods, to avoid the suffering of laboratory animals, as well as minimizing the risks of contamination of the final product by adventitious substances. However, in certain specific situations, the use of an *in vivo* system of production is unavoidable. This includes the following: (a) when the mAb concentrations are low ($< 5\mu g$/ml in batch systems, 50 μg/ml in hollow-fiber bioreactors, or 300μg/ml in membrane bioreactors, (b) when it has been proved that the use of cell culture results in a loss or decrease of the specific function of the mAb; (c) when the hybridoma line can only grow and synthesize the product *in vivo*; and (d) when the quantity to be produced exceeds the laboratory capacity of the producer (NAS, 1999). A noteworthy example of such a product generated *in vivo* is the therapeutic agent OKT3®, which can lose its biological activity when purified from the supernatant of a cell culture.

For antibodies produced *in vitro*, as is the case for many other proteins obtained from animal cell cultures, the main question involves the low level of expression of these products in the culture medium. This necessitates the use of large culture volumes for production, thus involving higher costs, especially for purification. In general, the optimization of these *in vitro* processes attempts to increase the concentration of the product in the medium. This is often possible by using high cell densities. Typical values for traditional processes are in the range of 20–100 mg/L, whereas for optimized systems, this can rise to 4.6 g/L (Kretzmer, 2002; Wurm, 2004).

It is unusual to find an optimized generic process for obtaining mAbs since each producer cell has a unique pattern of response to stress, consumption of nutrients and synthesis of products and byproducts. However, it is clear that the systems utilized for the production of mAb do not differ in any significant way from those using animal cells for the synthesis of other products, as can be seen in various chapters of this book (Chapters 5, 9, 11, and 12). For this reason, only those aspects that are especially relevant for obtaining mAbs from *in vitro* systems will be presented here.

17.5.1 Cell lines

Although various systems have been developed for the production of mAbs, only those involving cultures of animal cells will be presented in this chapter, since this is the system used to produce most of the antibodies on the market today (van Dijk and van de Winkel, 2001). Those of murine origin are produced directly from hybridomas, whereas the humanized or completely human ones come from the culture of animal cells transfected with specific genetic sequences, which are capable of following the patterns of glycosylation and the desired structural conformation, required for adequate drug performance.

Hybridomas constitute the most widely used cell lines for the production of mAbs, on both small and large scales. Section 17.4 of this chapter presents details of methods used to construct this type of cell. However, these cells produce antibodies that have limited therapeutic application,

due to the immunological responses to murine antibodies that often arise in patients.

As discussed above, one solution for this problem is the genetic manipulation of cell lines to enable them to synthesize humanized antibodies. In this situation, the hybridomas serve as an important source of gene sequences that codify antibody molecules (or their fragments), although these are later transfected into other animal cells. In general, the cell lines used are more robust than hybridomas, due either to their greater stability or even a reduced tendency for apoptosis.

The Chinese hamster ovary (CHO) cell line is now being used as a standard host cell for transfection with genes of interest for later use in the production of recombinant antibodies (Butler, 2005). Other cells with equivalent performance are the murine myelomas NS0 and Sp2/0, as well as baby hamster kidney (BHK), human embryonic kidney (HEK-93), and a derivative of the human retina (PER.C6®) (Chu and Robinson, 2001).

The selection of the best cell line should consider antibody productivity, as well as cell growth rate, although these parameters frequently follow opposite trends (Wurm, 2004). For the production of large amounts of mAb, it is fundamental that the productivity of the selected cell line is high. Otherwise, larger reaction volumes will be required, and the cost of purification will be increased. A reference value for specific protein productivity is 20 pg/cell per day.

Another important aspect involved in the selection of transfected lines is the capacity to grow without physical support, since the scale-up of such processes is much simpler than those designed for growth of anchorage-dependent cells. Thus, cells that grow naturally in suspension are preferred, such as myeloma cells (Sp2/0 and NS0), or others that can be easily adapted to this form of cultivation, such as CHO and BHK (Chu and Robinson, 2001).

17.5.2 Basic conditions for *in vitro* cultivation

Culture medium

Traditionally, the production of mAbs uses complex culture media containing glucose and amino acids as the main sources of carbon for cell metabolism, as well as vitamins, micronutrients and sometimes animal serum, usually fetal bovine serum. Chapter 5 provides a discussion on composition of culture media and recent trends in the search for formulas that do not require the use of animal serum, or of proteins of animal origin. These serum-free formulations use substitutes such as peptones, epithelial and fibroblast growth factors, hydrolysates, yeast extract, choline, and inositol. For the production of mAbs, various serum-free formulas are available, some of these developed specifically for a given cell line (Chu and Robinson, 2001). The development of those media is easier for non-anchorage-dependent cells, such as those used for mAb production. Thus, approximately 50% of the antibodies for therapeutic use are already produced using serum-free media. In some circumstances, the elimination of serum should be accompanied by the addition of other substances with the same shear stress protective effect of serum proteins,

such as Pluronic® F68 and carboxymethylcellulose (CMC) (Wu, 1995; Chu and Robinson, 2001).

Most of the cell culture media are formulated to attain a physiological osmolality in the range of 270–330 mOsm/kg. Under hypo-osmotic stress, hybridomas show a reduction in cell growth rate without any specific increase in antibody productivity (Ryu and Lee, 1999). However, under hyperosmotic stress, some hybridomas show physiological changes such as an increase in cell size, reduction in specific growth rate, and increase in specific mAb synthesis (Cherlet and Marc, 2000). The overall effect of these changes is a function of the cell line, and may or not result in an increase in the production of the antibody (Ozturk and Palsson, 1991; Bibila *et al.*, 1994). The addition of osmoprotectors such as glycine, betain, sarcosine, glycine, and proline may attenuate the toxic effects of high osmolality on growth, with an overall positive effect.

Influence of oxygen

Oxygen is crucial for the production of energy during phosphorylation and for the synthesis of cell components. In most cases, adequate growth conditions require an excess of dissolved oxygen (DO), with the optimum dependent on the cell line, cultivation conditions, and growth phase. The critical range of DO, that is, that which limits growth and/or mAb production, also varies as a function of cell line and cultivation conditions, and usually is in the range of 10–20% of air saturation.

The best system for aeration and stirring of culture medium in a bioreactor must minimize shear stress without significantly reducing the oxygen transfer (K_La) and yet avoiding the generation of foam. In some systems, protectors such as serum, Pluronic® F68, PEG, dextrans, lipids, and cholesterol, may have to be used to prevent shear stress. Sensitivity to shear depends on the cell line, and fortunately the lines most often utilized industrially for the production of mAbs, such as hybridomas, CHO, and BHK-21, are among the most resistant animal cells in relation to these hydrodynamic forces (Chisti, 2000; Chu and Robinson, 2001; Wu, 1995).

17.5.3 Cell metabolism

As discussed in Chapter 4, glucose and glutamine are the two main substrates in culture media used in animal cell processes, for generating intermediate components of anabolism and catabolism (Doverskog *et al.*, 1997). The fraction of the energy produced by the metabolism of each of the substrates depends on the cell line. Under conditions of excess glucose, the hybridomas normally obtain 90% of the ATP necessary for metabolic functions from glutamine, whereas CHO cells only obtain 40% (Jeong and Wang, 1995).

An excess of glucose and amino acids, especially glutamine, generates lactate and ammonia, respectively, and in many cases, these compounds inhibit the growth of animal cells and the formation of the products of interest, although the rate of mAb formation is not necessarily affected (Schneider *et al.*, 1996; Doverskog *et al.*, 1997). Large quantities of these metabolites in the culture medium can also diminish the quality of the

final product. Some cells can eliminate some of the metabolites by excreting them in the form of alanine, which is a non-toxic byproduct.

Lactate reduces the internal pH of the cells or of the culture medium, as well as increasing the osmolality of the medium. The mechanisms of toxicity of ammonia are less well understood (Schneider *et al.*, 1996). The critical concentrations of these compounds depend on the cell line and the culture conditions. Typical critical values for ammonia in the production of mAbs are in the range of 2–10 mM (Schneider *et al.*, 1996), whereas for lactate the range is much broader, varying from 1 to 300 mM.

17.5.4 Bioreactors and operation mode

Various combinations of bioreactors and operation mode have been used for the production of mAbs in several systems of expression, as shown in Chapter 9. All cells utilized for the production of mAbs grow in suspension. Those that did not initially have this capacity have been adapted (as is the case for CHO and BHK) (Butler, 2005). This results in a large number of options for production systems. Cells with this characteristic are easily cultivated in stirred-tank reactors, which have been scaled up to a volume of 10 000 L (Chu and Robinson, 2001; Kretzmer, 2002). This kind of bioreactor provides excellent homogeneity, facility for the implementation of control techniques, and the principles of scaling up are relatively well known. Other kinds of bioreactors for the production of mAbs are also available, such as air-lift, with volumes up to 1000 L, and also fixed-bed bioreactors (Moro *et al.*, 1994; Irving *et al.*, 1996; Kretzmer, 2002).

Products with less demand, such as those used in diagnosis, are developed in small-scale systems such as T-flasks, rollers, and hollow-fiber bioreactors (Kretzmer, 2002). The reduced size of these production systems makes it possible to operate various units in parallel to obtain different products. A small increase in scale can be reached by the multiplication of units.

Hollow-fiber bioreactors constitute an optimized production system where it is possible to achieve higher cell concentrations (10^7 to 10^8 cells/ml), and the product concentration can reach a level of 0.7–2.3 g/L, which is similar to what can be obtained with ascitic fluid (Hendriksen and Leeuw, 1998). This system can operate for over 3 months without affecting cell viability, but presents problems with mass transport, and the formation of nutrient gradients, which require specific solutions (Kretzmer, 2002).

The operation mode used in the industry is predominantly batch, given the simplicity and facility of control, good reproducibility, lower indices of contamination, and low production costs (Xie and Wang, 1997). However, this operation mode has some disadvantages, the low cell and product concentration, because it permits the accumulation of ammonia and lactate, or the exhaustion of essential nutrients (Xie and Wang, 1997, 2006). Typical antibody concentration values in a batch system using a stirred-tank bioreactor or air-lift are in the range of 100μg/ml.

Two approaches are possible to maximize the concentration of antibodies in the culture. The first is based on an increase in the specific

production rate by adding epidermal or fibroblast growth factors, IL-2 (Jang and Barford, 2000), butyric acid (Cherlet and Marc, 2000), or cyclic nucleotides (Dalili and Ollis, 1988). Moreover, the addition of thymidine, the reduction or elimination of serum, suboptimal concentrations of oxygen, a reduction in pH, and the increase in osmolarity are situations that can disturb growth, resulting in an increase in the specific antibody production rate (Ozturk and Palsson, 1991; Reddy *et al.*, 1992).

The second focus is based on obtaining greater cell concentrations for longer time periods to maximize the production of mAbs (Duval *et al.*, 1991; Bibila and Robinson, 1995). In general, at least one of the following factors would discourage growth and/or production: (a) exhaustion of nutrients, especially certain essential amino acids, vitamins, glucose or serum (Glassy *et al.*, 1988; Duval *et al.*, 1991; Jo *et al.*, 1993a, 1993b; Hiller *et al.*, 1994); (b) inhibition due to the formation of toxic byproducts, such as lactate and ammonia (Ozturk *et al.*, 1992); and (c) inadequate concentration of dissolved oxygen. Satisfactory results can be obtained simply by increasing the concentration of nutrients and promoting an equilibrium of salts to guarantee adequate osmolality (Jo *et al.*, 1993a). However, there is no way of avoiding the constant accumulation of inhibiting metabolites, which ends up leading the process to collapse.

The fed-batch operation mode has been widely used in the industry due to the simplicity of operation and control possibilities, easy scaling-up, and efficiency in obtaining high cell and product concentrations (Jo *et al.*, 1993b; Bibila and Robinson, 1995; Xie and Wang, 1997; Sauer *et al.*, 2000). The increase in productivity is due to the manipulation and exploration of the physiological state of the cells, redistributing the available resources and rerouting the flow of metabolites. This avoids the synthesis of toxic byproducts and, consequently, prolongs the phase of product accumulation. However, it is inevitable that a progressive reduction in viability will arise from problems of accumulation of toxic byproducts and high osmolality.

The continuous process is the only way of avoiding the accumulation of inhibitors, since it guarantees their constant removal at the same time as it supplies the system with the nutrients necessary for the performance of the cells. If perfusion is used, with cell retention, the system provides a high cell density that is controllable, without gradients, and which can maintain optimal conditions of nutrient concentration and toxic byproducts.

References

Abbas AK, Lichtman AH, Pober JS (2000), Cellular and Molecular Immunology, 4th Ed., WB Saunders, Philadelphia.

Bibila T, Robinson DK (1995), In pursuit of the optimal fed batch process for monoclonal antibody production, Biotechnol. Prog. 11:1 13.

Bibila T, Glazomitsky K, Ranucci CS, Robinson DK, Silberklang M, Buckland BC, Aunins JG (1994), Monoclonal antibody process development using medium concentrates, Biotechnol. Prog. 10:87 96.

Butler M (2005), Animal cell cultures: recent achievements and perspectives in the production of biopharmaceuticals, Appl. Microbiol. Biotechnol. 68:283 291.

Cherlet M, Marc A (2000), Stimulation of monoclonal antibody production of hybridoma cells by butyrate: evaluation of a feeding strategy and characterization of cell behavior, Cytotechnology 32:17 29.
Chisti Y (2000), Animal cell damage in sparged bioreactors, Trends Biotechnol. 18:420 432.
Chu L, Robinson DK (2001), Industrial choices for protein production by large scale cell culture, Curr. Opin. Biotechnol. 12:180 187.
Cotton RG, Milstein C (1973), Fusion of two immunoglobulin producing myeloma cells, Nature 244:42 43.
Dalili M, Ollis DF (1988), The influence of cyclic nucleotides on hybridoma growth and monoclonal antibody production, Biotechnol. Lett. 10:781 786.
De St Groth FS, Scheidegger D (1980), Production of monoclonal antibodies: strategies and tactics, J. Immunol. Methods 35:1 21.
Doverskog M, Ljunggren J, Ohman L, Haggstron, L (1997), Physiology of cultured animal cells, J. Biotechnol. 59:103 115.
Duval D, Demangel C, Jolain KM, Miossec S, Geahel I (1991), Factors controlling cell proliferation and antibody production in mouse hybridoma cells: I. Influence of the amino acid supply, Biotechnol. Bioeng. 38:561 570.
Emanuel PA, Dang J, Gebhardt JS, Aldrich J, Garber EAE, Kulaga H, Stopa P, Valdes JJ, Dion Schultz A (2000), Recombinant antibodies: a new reagent for biological agent detection, Biosens. Bioelectron. 14:751 759.
Falkenberg FW (1998), Monoclonal antibody production: problems and solutions, Res. Immunol. 149:542 547.
Glassy MC, Tharakan JP, Chau PC (1988), Serum free media in hybridoma culture and monoclonal antibody production, Biotechnol. Bioeng. 32:1015 1028.
Hendriksen CFM, Leeuw W (1998), Production of monoclonal antibodies by the ascites method in laboratory animals, Res. Immunol. 149:535 542.
Hiller GW, Clark DS, Blanch HW (1994), Transient response of hybridoma cells in continuous culture to step changes in amino acid and vitamin concentrations, Biotechnol. Bioeng. 44:303 321.
Horibata K, Harris AW (1977), Mouse myelomas and lymphomas in culture, Exp. Cell Res. 60:61 77.
Hudson PJ, Souriau C (2003), Engineered antibodies, Nat. Med. 9:129 134.
Huhalov A, Chester KA (2004), Engineered single chain antibody fragments for radioimmunotherapy, Q. J. Nucl. Med. Mol. Imag. 48:279 88.
Irving RA, Hudson PJ, Goding JW (1996), Construction, screening and expression of recombinant antibodies, In: Goding JW (Ed.), Monoclonal Antibodies: Principles and Practice, 3rd Ed., Academic Press, Manchester, pp. 424 464.
Jang JD, Barford JP (2000), Effect of feed rate and antibody production in the fed batch culture of murine hybridoma cells, Cytotechnology 32:229 242.
Jeong YH, Wang SS (1995), Role of glutamine in hybridoma cell culture: effects on cell growth, antibody production, and cell metabolism, Enzyme and Microbial Tech nology 17:47 55.
Jo EC, Kim DI, Moon HM (1993a), Step fortifications of nutrients in mammalian cell culture, Biotechnol. Bioeng. 42:1218 1228.
Jo EC, Park HJ, Kim DI, Moon HM (1993b), Repeated fed batch culture of hybridoma cells in nutrient fortified high density medium, Biotechnol. Bioeng. 42:1229 1237.
Karpas A, Dremucheva A, Czepulkowski B H (2001), A human myeloma cell line suitable for the generation of human monoclonal antibodies, Proc. Natl Acad. Sci. U S A 98:1799 1804.
Köhler G (1981), The technique of hybridoma production, In: Immunological Meth ods, vol. II, Academic Press, pp. 285 308.
Köhler G, Milstein C (1975a), Continuous cultures of fused cells secreting antibody of

predefined specificity, Nature 256:495 497.
Köhler G, Milstein C (1975b), Derivation of specific antibody producing tissue culture and tumor lines by cell fusion, Eur. J. Immunol. 6:511 519.
Köhler G, Shulman MJ (1978), Cellular and molecular restrictions of the lymphocyte fusion, Curr. Top. Microbiol. Immunol. 81:143 148.
Kretzmer G (2002), Industrial processes with animal cells, Appl. Microbiol. Biotechnol. 59:135 142.
Lin MZ, Teitell MA, Schiller GJ (2005), The evolution of antibodies into versatile tumor targeting agents, Clin. Cancer Res. 11:129 38.
Little M, Kipriyanov SM, Le Gall F, Moldenhauer G (2000), Of mice and men: hybridoma and recombinant antibodies, Immunol. Today 21:364 370.
Littlefield JW (1964), Selection of hybrids from matings of fibroblasts in vitro and their presumed recombinants, Science 145:709 710.
Maki R, Traunecker A, Sakano H, Roeder W, Tonegawa S (1980), Exon shuffling generates an immunoglobulin heavy chain gene, Proc. Natl Acad. Sci. U S A 77:2138 2142.
Moro AM, Rodrigues MTA, Gouveia MN, Silvestri MLZ, Kalil JE, Raw I (1994), Multi parametric analyses of hybridoma growth on glass cylinders in a packed bed bioreactor system with internal aeration. Serum supplemented and serum free media comparison for MAb production, J. Immunol. Methods 176:67 77.
NAS (1999), Monoclonal Antibody Production, National Academic Press, Washington, DC.
Nelson PN, Reynolds GM, Waldron EE, Ward E, Giannopoulos K, Murray PG (2000), Demystified monoclonal antibodies, J. Clin. Pathol. Mol. Pathol. 53: 111 117.
Ozturk SS, Palsson BO (1991), Growth, metabolic, and antibody production kinetics of hybridoma cell culture: 2. Effects of serum concentration, dissolved oxygen concentration, and medium pH in a batch reactor, Biotechnol. Prog. 7:481 494.
Ozturk SS, Riley MR, Palsson BO (1992), Effects of ammonia and lactate on hybridoma growth, metabolism, and antibody production, Biotechnol. Bioeng. 39:418 431.
Pernis B, Chiappino G, Kelus AS, Gell PGH (1965), Cellular localization of immunoglobulins with different allotypic specificities in rabbit lymphoid tissues, J. Exp. Med. 122:853 877.
Reddy S, Bauer KD, Miller WM (1992), Determination of antibody content in live versus dead hybridoma cells: analysis of antibody production in osmotically stressed cultures, Biotechnol. Bioeng. 40:947 964.
Reichert J, Pavlou A (2004), Monoclonal antibodies market, Nat. Rev. 3:383 384.
Roque ACA, Lowe CR, Taipa MA (2004), Antibodies and genetically engineered related molecules: production and purification, Biotechnol. Prog. 20:639 654.
Ryu JS, Lee GM (1999), Application of hypoosmolar medium to fed batch culture of hybridoma cells for improvement of culture longevity, Biotechnol. Bioeng. 62:120 123.
Sakano H, Maki R, Kurosawa Y, Roeder W, Tonegawa S (1980), Two types of somatic recombination are necessary for the generation of complete immunoglobulin heavy chain genes, Nature 286:676 83.
Sauer PW, Burky JE, Wesson MC, Sternard HD, Qu L (2000), A high yielding, generic fed batch cell culture process for production of recombinant antibodies, Biotechnol. Bioeng. 67:565 597.
Schneider M, Marison IW, von Stockar U (1996), The importance of ammonia in mammalian cell culture, J. Biotechnol. 46:161 185.
Shulman M, Wilde CD, Köhler G (1978), A better cell line for making hybridomas secreting specific antibodies, Nature 276:269 270.
Steinitz M, Klein JG, Koskimies S, Makel O (1977), EB virus induced B lymphocyte

cell lines producing specific antibody, Nature 269:420 422.
Tonegawa S, Maxamt AM, Tizardt R, Bernard O, Gilbertt W (1978), Sequence of a mouse germ line gene for a variable region of an immunoglobulin light chain, Proc. Natl Acad. Sci. U S A 75:1485 1489.
Vaisbourd M, Ignatovich O, Dremucheva A, Karpas A, Winter G (2001), Molecular characterization of human monoclonal antibodies derived from fusions of tonsil lymphocytes with a human myeloma cell line, Hybrid Hybridomics 20:287 92.
van Dijk MA, van de Winkel JGJ (2001), Human antibodies as next generation therapeutics, Curr. Opin. Chem. Biol. 5:368 374.
Vijayalakshmi MA (1989), Pseudobiospecific ligand affinity chromatography, Trends Biotechnol. 7:71 76.
Watson JD, Gilman M, Witkowski J, Zoller M (1998), Recombinant DNA, 2nd Ed., Scientific American Books, New York.
Wu J (1995), Mechanisms of animal cell damage associated with gas bubbles and cell protection by medium additives, J. Biotechnol. 43:81 94.
Wurm FM (2004), Production of recombinant protein therapeutics in cultivated mammalian cells, Nat. Biotechnol. 22:1393 1398.
Xie L, Wang DIC (1997), Integrated approaches to the design of media and feeding strategies for fed batch cultures of animal cells, Trends Biotechnol. 15:109 113.
Xie L, Wang DIC (2006), Fed batch cultivation of animal cells using different medium design concepts and feeding strategies, Biotechnol. Bioeng. 95:270 284.

Viral vaccines: concepts, principles, and bioprocesses

18

Isabel Maria Vicente Guedes de Carvalho Mello, Mateus Meneghesso da Conceição, Soraia Attie Calil Jorge, Pedro Estilita Cruz, Paula Maria Marques Alves, Manuel José Teixeira Carrondo, and Carlos Augusto Pereira

18.1 Introduction

Animal cell culture technology has always played a major role in the development of virology. Significant progress in the propagation of viruses and the consequent development of viral vaccines were only made possible after the 1950s, upon the establishment of cell culture technology. Animal cell culture gradually substituted live animals in the preparation of viral antigens used in vaccination, such as the vaccines against smallpox and rabies. At the same time, the production of viral antigens in cell cultures led to considerable progress in bioprocesses technology. Cell culture bioprocesses are now well established in bioreactors of up to 12 000 L.

Recent developments in virology and cell culture technology have allowed research and development laboratories to engage in molecular manipulation of viruses and cells for bioprocess production of viral gene products. Examples are the establishment of recombinant viral vectors for expression in animal cells that have significant potential in producing recombining vaccines and treatment by gene therapy. Thus, a recombinant vaccine against hepatitis B virus (HBV) has been developed and several others are in the final phases of clinical trials.

This chapter concerns the development of cell culture technology for viral vaccine production, which is related to: (a) a need for prophylaxis and/or treatment of the most important viral diseases, such as AIDS, hepatitis C, influenza, and papillomavirus; (b) the establishment of current molecular technologies, and (c) a reduction in risk factors for the manipulation of live viral particles.

Cell culture technology for the production of viral products is focused on establishing protocols for recombinant products of low risk to prevent viral diseases. In many countries, viruses are increasingly important as biological agents for the control of agricultural pests (see Chapter 19).

18.2 Viral replication

The pathological effects of viral diseases are the consequence of various factors, such as the toxicity of the products of metabolism of infected cells and host reaction to infected cells expressing viral protein, as well as changes in the genetic expression of host cells through structural or functional interactions with genetic material or viral proteins. In some cases, the symptoms or acute signs of diseases caused by a virus can be directly related to the elimination of the infected cells.

For a better understanding of the pathological effects caused by viral infections and of their control by vaccination or antiviral therapy, it is important to understand how viruses infect cells, express their genes, multiply, and change the cellular metabolism after the infection. The genetic characteristics of the host as well as its sensitivity, are factors that must be considered in evaluating the magnitude of viral replication.

Viruses are exclusively intracellular organisms and therefore depend on the cells to multiply. A complete viral particle, or virion, consists of one nucleic acid molecule (RNA or DNA) covered by a protein layer (nucleocapsid). Some virions have a lipid cover with a glycoprotein envelope. The main function of the virion is to transport the viral genome to the interior of the host cell to be replicated and amplified.

The infectivity of host cells varies considerably between viruses. A specific virus may have a great diversity of host cells, while another may be capable of infecting only one type of cell. The sensitivity defines the capacity of the cell or animal to be infected.

Viral multiplication involves different ways of replication. However, there are some common characteristics in the replicative cycles of viruses. Initially, viruses insert their genetic material (RNA or DNA) into the cell, and the size, composition, and genetic organization of this material vary significantly between viruses, as well as the proteins that are needed for replication. After the infection, there is a period called the eclipse phase, when only few viruses are found in the infected cells. During this phase, the genome and all the viral machinery is exposed to the host, but the viral progeny is still small. Afterwards, there is a pause when virions accumulate inside or outside of the cell at an exponential rate. This pause is called the maturation phase. After some hours, lytic viruses cause cellular lysis with the cessation of all metabolic activity and the cells lose their structural integrity. Cells infected by non-lytic viruses can continue virion synthesis over a long period of time.

The reproductive cycle of viruses may take hours or days. The infection of cells does not guarantee the production of viral progeny, which may be productive, restricted, aborted, or latent. A productive infection occurs in permissible cells and results in infectious viral particles. An abortive infection may occur in two circumstances: firstly, although the cell is sensitive to infection, it is not necessarily permissive, allowing the expression of only a few viral genes. The second circumstance is when a sensitive cell, permissive or not, is infected by defective viruses that do not have all the necessary viral genes for their replication. Furthermore, the cells can be temporarily permissible. In this case, the viral particles may remain in the cells until they become permissive or else some viral particles may be

produced for a limited period of time by a fraction of the cell population. This type of infection is called restricted. In a latent infection, the viral genome persists in temporarily permissive cells without the destruction of the infected cells.

The following sections outline the stages of the replication used by viruses.

18.2.1 Adsorption

This stage consists of the virus binding to the cell. This involves the specific binding of a glycoprotein that appears on the external structure of a virus to a host cell receptor.

The sensitivity of a cell to a specific virus is frequently related to the presence of these receptors in the cell membrane. The concept of sensitivity is not related to permissiveness. A cell may not be sensitive to a certain virus due to lack of receptor, but may produce viral progeny if its viral genome is introduced into its cytoplasm.

18.2.2 Internalizing and unwrapping the viral particle

After the binding, the virus may use various means of penetration: (a) direct penetration, via the translocation of the entire virus through the cytoplasmic membrane; (b) endocytosis, which is mediated by receptors, resulting in the formation of intercytoplasmic vesicles containing many viral particles; (c) direct fusion of the viral envelope with the cytoplasmic membrane.

Non-enveloped viruses generally use the first two penetration mechanisms, while the enveloped viruses enter a cell by endocytosis followed by binding with the membrane of an endosome. In addition to this mechanism, the enveloped viruses fuse directly with the cell membrane. The fusion of the viral envelope with the cell membrane requires the interaction of the glycoproteins of the virus with a cell receptor. After the internalization of the viral particle, the genome is freed for later expression. This process is known as unwrapping and it involves both cellular and viral enzymes.

18.2.3 Structure and organization of viral genomes

Viral genomes consist of RNA or DNA, which can be single- or double-stranded, and may consist of one or more fragments. During viral replication, both DNA and RNA viruses synthesize protein by translation of messenger RNA. The mRNA is then translated by the cell into the viral proteins that will constitute the viral particles.

Virus with single-strand RNA

There are three groups of virus with a single-stranded RNA genome (ssRNA). The first group comprises viruses with RNA that functions both as genomic and messenger (for example, picornovirus and flavovirus). The ssRNA in these viruses is conventionally called positive-strand virus

(+sRNA). After it enters the cell, the +sRNA functions as mRNA, binding with cellular ribosomes to complete its translation into proteins. The product of this translation is a polyprotein that will later be cleaved. The second function of this RNA is genomic, to be used as a precursor for the synthesis of a complementary negative RNA strand (RNA–) catalyzed by a polymerase derived from the polyprotein cleavage. The negative strand will be transcribed once again as genomic RNA (RNA+) through a viral polymerase. During this process, several strands of genomic RNA will be produced, as well as proteins that will be used to produce the viral particles. One characteristic of the RNA+ virus replication is the capacity of its genomic RNA to function as mRNA after the infection, thus leading to the synthesis of the enzymes responsible for the viral genome replication, without need for the complete viral particle to carry the enzymes (*Figure 18.1*). This allows the RNA extracted from the virus to become infectious, although less than the complete viral particle.

Another group of ssRNA viruses consists of those that have negative single-strand RNA (–sRNA). This group consists of orthomyxovirus, paramyxovirus, and rhabdovirus, among others. The genomic RNA of these viruses has two functions: to serve as a template for transcription and for replication. The viral genome must translate its own mRNA in order to synthesize viral proteins because the host cell does not have the appropriate enzymes. Therefore the –sRNA virus will have a transcriptase along with its genome. The isolated viral genome is not infectious, because there must be a viral transcriptase to translate the genomic RNA into several mRNA strands that will later be translated into viral proteins. These are used to make a copy of all its genome, producing a large strand

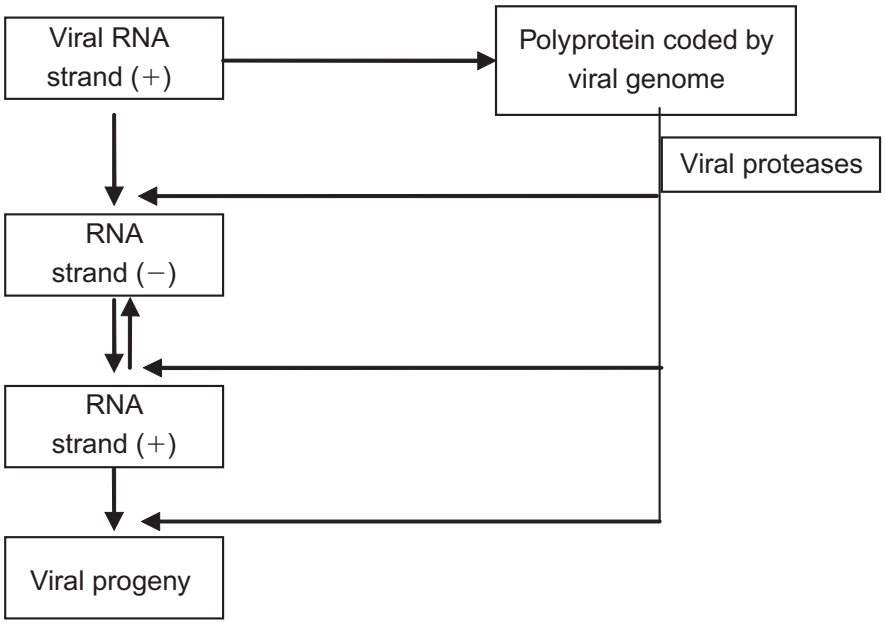


Figure 18.1

Flow of events during the replication of a positive strand RNA virus.

of +mRNA that will serve as a template for the synthesis of genomic – RNAs (*Figure 18.2*).

The retroviruses are the third group of single-strand RNA virus. These viruses have a more complex strategy for the production of mRNA. The retroviruses have a diploid genome associated with a DNA-dependent RNA polymerase (reverse transcriptase) that transcribes RNA into a hybrid DNA–RNA. The RNA strand is digested by the viral ribonuclease H and a complementary DNA is synthesized, thus producing a double-strand DNA (dsDNA) that will be inserted into the cellular genome with the participation of the viral integrase enzyme, producing a provirus (*Figure 18.3*). After the integration, the cellular-dependent polymerase RNA–DNA will initiate the synthesis of the proteins that are essential to replicate the viral genome followed by the synthesis of proteins to produce the virus.

Virus with double-strand RNA

The genome of reoviruses is formed by double-strand RNA (dsRNA) composed of several dsRNA fragments (10–12). The RNA fragments are transcribed inside a capsid by a polymerase, giving rise to several mRNAs. Here, the mRNA molecules have two functions: (a) they are translated as monocistronic messengers, forming viral proteins; (b) they serve as a template for the synthesis of a complementary RNA strand, giving rise to double-strand RNA genomic fragments (*Figure 18.4*).

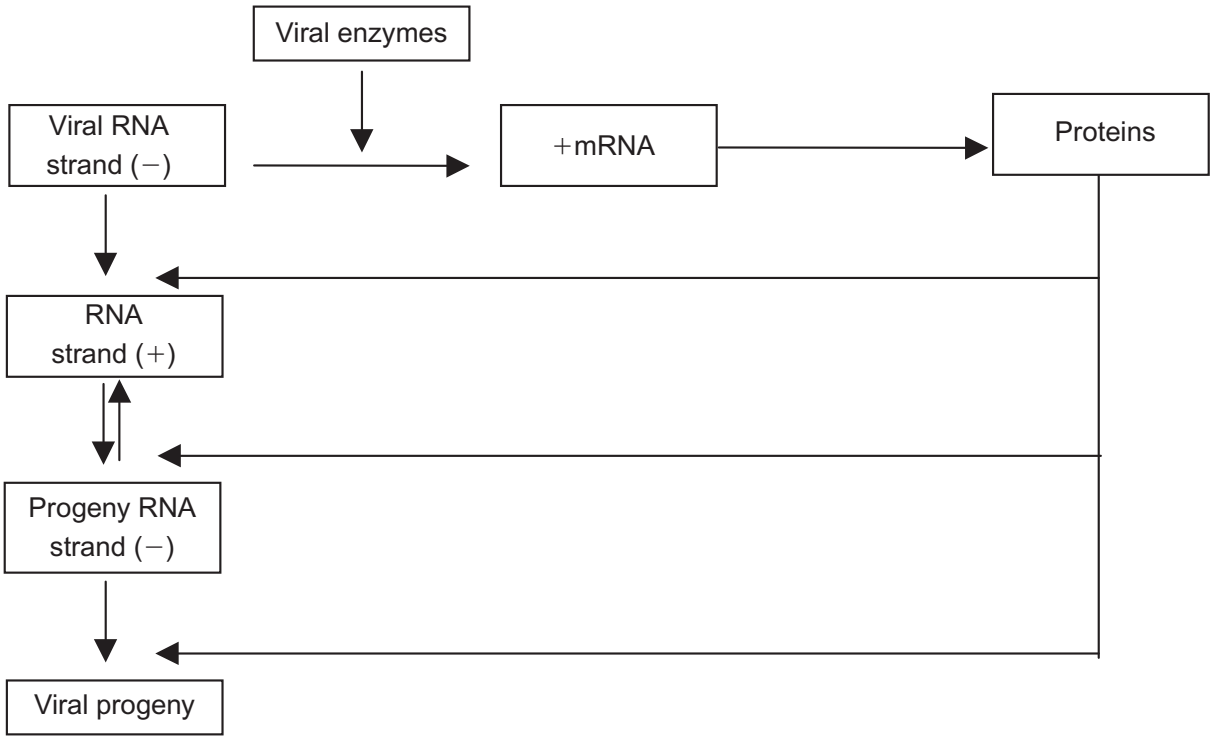


Figure 18.2

Flow chart of events during the replication of negative single strand RNA virus.

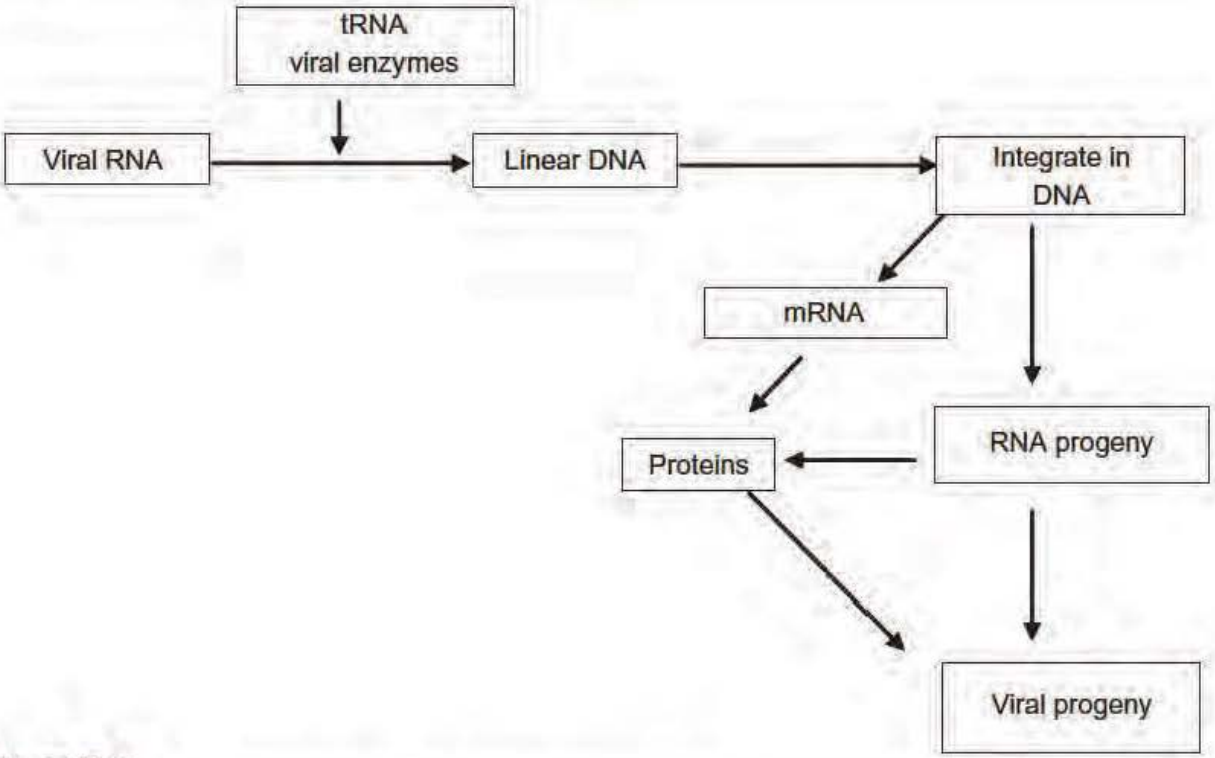


Figure 18.3

Flow chart of events during the replication of retroviruses.

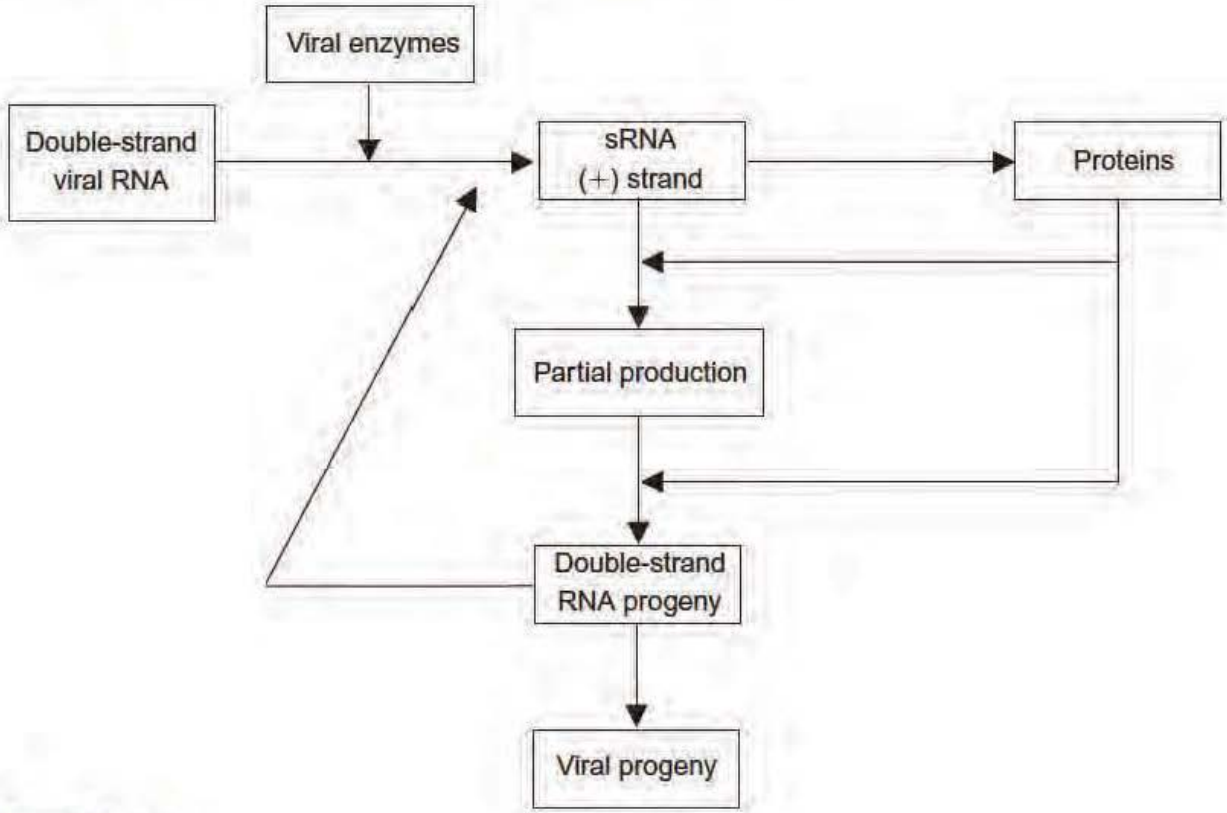


Figure 18.4

Flow chart of events during the replication of reovirus.

Virus with DNA

The double-strand DNA viruses are transported into the nucleus where they translate and replicate their genome using cellular enzymes to produce mRNA. These mRNA strands are later translated into proteins that direct the synthesis of the proteins and genomes of the virus. The isolated DNA of these viruses is considered infectious (*Figure 18.5*). There are some double-strand DNA viruses, such HBV, which have a different replication strategy that includes an intermediary RNA and a reverse transcription phase. In this case, after the viral DNA penetrates the nucleus of the cell, it is converted into a covalently closed circular DNA molecule (cccDNA). Before the viral genome replication occurs, a cellular enzyme (DNA-dependent RNA II polymerase) translates several RNAs from cccDNA and genomic and subgenomic RNAs (*Figure 18.5*). Most DNA viruses replicate in the nucleus of the cell, except for poxviruses, which replicate in the cytoplasm. These viruses are practically autonomous in terms of translation factors. Parvoviruses have a single-strand DNA genome and replicate inside the cellular nucleus using replicative-cycle cellular polymerases.

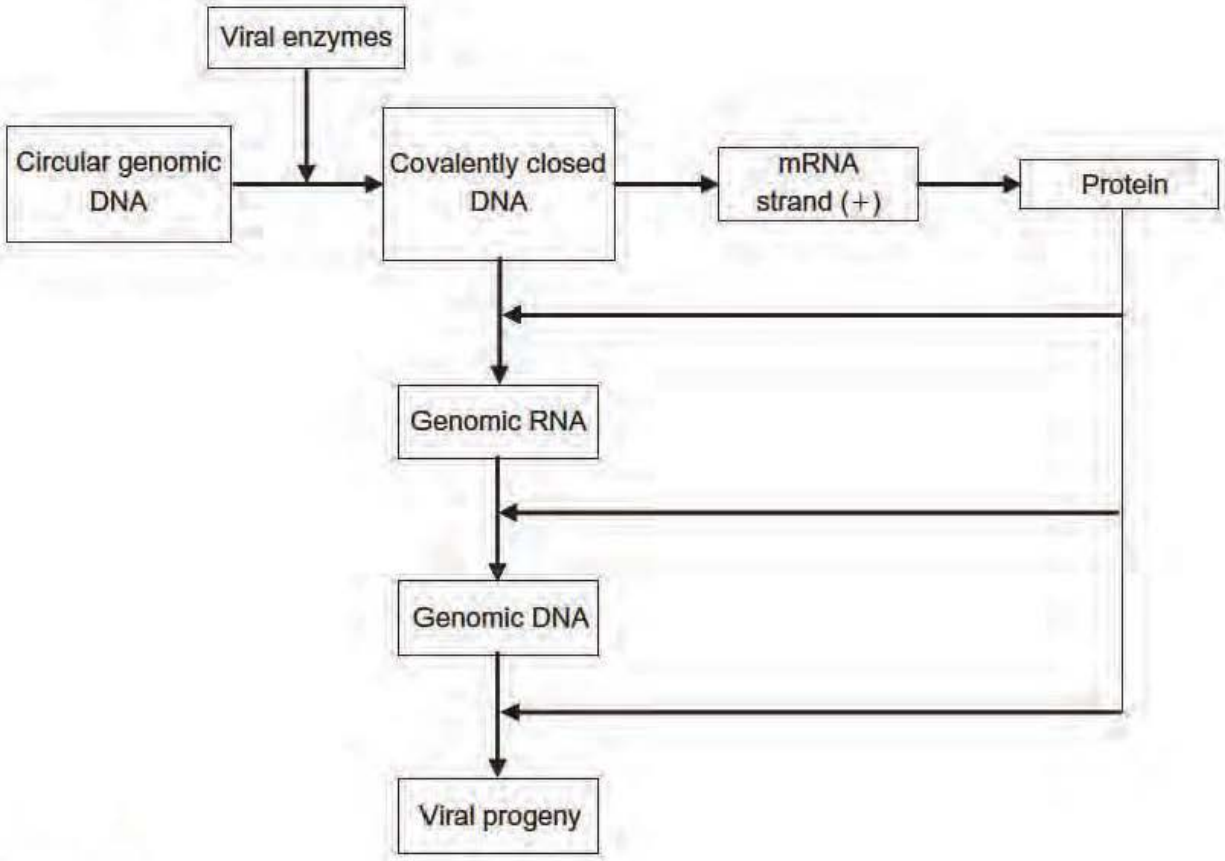


Figure 18.5

Flow chart of events during the replication of the hepatitis B virus.

18.2.4 Production and maturation of viral particles

Viruses use three strategies to assemble, mature, and release from infected cells. With the first strategy, represented by picornavirus and adenovirus, the assembly and maturation are completed inside the cell, either in the cytoplasm or in the nucleus. As a rule, all non-enveloped viruses are produced and become infectious inside the cell and depend on the cellular lysis for release.

The second strategy is used by enveloped viruses, such as togavirus and retrovirus, which are enveloped in the cytoplasmic membrane. They are generally released by the budding of cellular membranes or exocytose through a vacuole, usually without cellular lysis.

The third strategy is used by viruses whose nucleocapsids are produced in the nucleus of the cell, with assembly and maturation involving the nuclear membrane. The enveloped viruses accumulate in the endoplasmic reticulum and are carried to the cell surface via vacuoles. Some viruses that use this process, such as herpesvirus, are cytolytic (cause cell lysis).

Knowledge of viral replication strategies has helped to optimize the approaches for the genetic modification of the viruses for better expression of the heterologous genes. It has also led to the introduction of bioprocesses for the production of infectious viral particles or viral-like particles, as will be discussed next.

18.3 Production of viral particles by cell culture

Viral particle production from cell cultures has several differences from other bioprocesses. The production of molecules like enzymes, toxins, or other proteins synthesized by bacteria, fungi or animals, depend upon culture parameters, such as pH, temperature, dissolved oxygen, or nutrients. Product formation may occur through secondary metabolic pathways, which are not related to the development or growth of the cell. In these situations, research and technological development must be directed to the specific cell and this involves the improvement of the cell as a better molecular production unit. So, there is a direct relation between nutrient conversion, cell growth, and the expected improvement of the final productivity.

Viral particle production processes by cell culture infection, cannot be characterized in such a simple way, since the final product – ‘‘virus’’ – does not result from a secondary metabolic pathway. However, it can be better described as a process redirecting the cell machinery towards viral particle production, which only happens after viral infection. The virus production process can be divided into two different steps. The first involves cell multiplication, which results from the conversion of culture medium substrates into cell mass. At the instant of viral infection, the cellular production unit no longer exists, since the viral genetic material forms a new production unit, initiating the second step of the virus production process. This production unit is the infected cell and is the producer of new viral particles. This production phase requires nutritional and metabolic conditions that are not observed during cell growth. These conditions are normally studied separately. Nevertheless, virus production

essentially requires the development of the cell that is ''consumed'' during the virus production phase, and the metabolic status of the cell at the moment of infection is a key factor for the success of the viral particle production process.

Industrial production of viral vaccines preferably requires the use of continuous or immortalized cell lines as the basis for viral multiplication. BHK-21, Vero, MDCK, MDBK, CHO, or HeLa cells are the main platforms used in the production of a huge variety of viral vaccines, since they are considered stable, do not suffer significant genetic modifications even after numerous generations of growth, and have a great capacity for *in vitro* growth. In addition, these cell lines are susceptible to infection by several different viruses, irrespective of their origin. As an example, the Vero cell line has been obtained from an African green monkey kidney. Although this cell immortalization process results in metabolic and functional changes not observed in normal cells, Vero cells still preserve some typical kidney cell characteristics. Despite this, certain viruses, like rabies or poliovirus, which naturally infect nervous tissues, are easily adapted to infect Vero cells (Frazzatti-Galina *et al*., 2001). The multifunctional property of continuous cell lines is important for establishing the best platforms for an industrial production process.

Even though there are many cell lines capable of cultivation in suspension, the majority of cells isolated from animal tissues retain their adherent physiological characteristics and must be grown on a solid surface. These are named adherent cells.

The basic need for a solid support guides all production choices involving industrial processes for adherent cells. A large variety of vessels has been developed for adherent cell cultures. Petri dishes, Roux bottles, T-flasks, and roller bottles are examples of cell culture vessels with a glass or polystyrene surface. The system of choice is dependent on the scalability of multiple steps, as well as the cost of equipment and qualified operators.

As mentioned in Chapter 9, since production scale-up is related to the increase of cell culture surface for adherent cells, consideration must be given to the relationship between the surface area available for cell growth and the bioreactor volume (Kent and Mutharasani, 1992).

The first adherent cell culture industrial process was conducted in roller bottles, a system with limited scale-up possibilities. However, a great advance in scale-up of high density cultures was achieved by Van Wezel in 1967. This allowed the culture of adherent cell lines on the surface of microspheres, called microcarriers. Even under low agitation conditions, microcarriers remain suspended in medium, which allows the culture to be homogeneous and to be controlled easily to maintain optimal physiological conditions, such as pH, temperature, and aeration. Besides, this is the only system for adherent cells that allows constant monitoring of cell growth. This enables cell morphology to be monitored and combines all the advantages of suspension culture systems with the requirements of adherent cells (Griffiths *et al*., 1987). So, the process can be well monitored and controlled with high density cell cultures obtained in small bioreactor volumes.

Vaccine production based on cells can use different methods of culture.

A classic way to produce viral vaccines consists of cultivating cells on an appropriate static support, infecting them with virus, collecting and purifying the virus produced and formulating the vaccine.

Although many adherent cell lines have already been adapted to cultivation in suspension, a large number of viral infection agents do not often allow the usage of those cells as a platform for prompt vaccinal antigen production. For this reason, overcoming roller bottles scale limits, microcarrier systems have been developed as a faithful technological alternative for simplifying the adherent cell scale-up process.

Nahapetian *et al.* (1986) obtained high density Vero cells cultures that reached 3×10^7 cells/ml, when using a microcarriers system at a perfusion rate of eight volumes of medium per day, an amount 10 times higher than that usually obtained with repeated batch cultures. Similar results have been described by Mendonça *et al.* (1993, 1999, 2001) who also obtained high density Vero cell cultures when using perfusion rates of 3 volumes of medium per day. According to Nahapetian *et al.* (1986), endogenous synthesis of cellular growth factors is probably the growth-limiting factor of under-fed cultures, while factors related to culture conditions such as aeration and cell metabolite production are involved in the growth limitation of over-fed cultures.

Table 18.1 compares the relationship between cell culture surface area and bioreactor volume in many different culture systems usually used with adherent cells. For microcarriers, this coefficient might reach 60 cm^2/ml of medium for culture area prepared with 10 mg of microcarriers per milliliter. For Roux bottles, this coefficient is around 3 cm^2/ml. In cultures initiated with 2 mg of microcarriers per milliliter of medium, high cell densities of even 3×10^6 cells/ml are often reached, compared with smaller cell densities from 2 to 3×10^5 cells/ml usually observed in Roux bottle systems. Another great advantage of the use of microcarrier culture systems is the possibility of preparing cell cultures with hundreds or even thousands of liters (Montagnon *et al*., 1984).

According to Butler (1987), many materials have been used in the production of microcarrier particles, and they have specific requirements that allow appropriate cell adherence and growth:

(i) particle density should be between 1.02 and 1.05 g/cm^3 to facilitate their maintenance in suspension at low agitation rates (40–150 rpm);

Table 18.1 Relationship between cell culture surface area and bioreactor volume used with animal adherent cells

Cell culture systems	Area/volume (cm^2/cm^3)
Roller bottle	0.2 0.7
Cell factories	1.7
Plastic bags	5.6
Fixed bed	10 15
Hollow fiber	31
Cytodex™ microcarriers (10 g/l)	34
CultiSpher® G microcarriers (30 g/l) LF	120

(ii) particles should preferably be transparent, allowing microscopic evaluation;
(iii) rigid materials (polystyrene and glass) are recommended because of their low porosity;
(iv) surface charge can be positive or negative, but it should not be too low because of the risk of difficulties of cell adherence, and it should not be too high because it could inhibit cell growth; the charge should be equally distributed throughout the surface to insure homogeneous cell distribution.

Microcarrier particle diameter should ideally be between 100 and 400 μm with a size distribution of ± 25 μm to guarantee a homogeneous culture. According to Butler and Spier (1984), cells tend to adhere preferably to the smallest particles. However, Hu and Wang (1987) demonstrated that higher microcarrier diameter tended to promote cell growth. An increase of diameter from 185 to 265 μm resulted in a longer exponential growth phase, with a final cell concentration four times higher than that observed with microcarriers with smaller diameter.

Adherent cells attached to microcarriers are particularly susceptible to damage caused by mechanical shear forces within agitated tanks. This vulnerability to damage is usually associated with cell immobilization and with the increase in shear force sensitivity of suspended microcarriers. Croughan *et al.* (1987) demonstrated that FS-4 and Vero cells are highly sensitive to shear forces caused by an increase in the mechanical agitation of the cell culture. They showed a progressive reduction in cell growth with higher agitation velocities and also cell lysis at an agitation rate over 180 rpm.

A great variety of mammal, bird, fish, amphibian, and insect cells can be cultivated in this system. To ensure that cells from so many different organisms and tissues can be cultivated with success in microcarriers, these microcarriers should have a physicochemical composition and other specific characteristics appropriate to the specific cell line. The most commonly used microcarriers are composed of DEAE-dextran polymers, polyacrylamide, polystyrene, gelatin, or glass. These present a great variety of density, size, weight, or electric charge that might have a significant effect in cell culture (Varani *et al*., 1983; Reuveny *et al*., 1985). Some examples are given in *Figure 18.6*.

Many different culture systems have demonstrated effectiveness for viral production. As previously described, these systems are based on the growth of cells in suspension or adherent to microcarriers, which are kept in suspension by agitation. After achieving high density the cultured cells can be infected by virus, allowing intracellular viral multiplication until the viral products are finally collected and processed. After standardization and optimization, these systems allow consistent viral particle production, and these steps are called the synthesis or upstream phase. *Figure 18.7* shows a typical cell membrane structure when rabies viral particles are leaving the surface of an infected cell.

For viral vaccine preparation or downstream processing, the cells and supernatant of infected cultures should go through concentration and purifying processes. These are important steps because there could be a

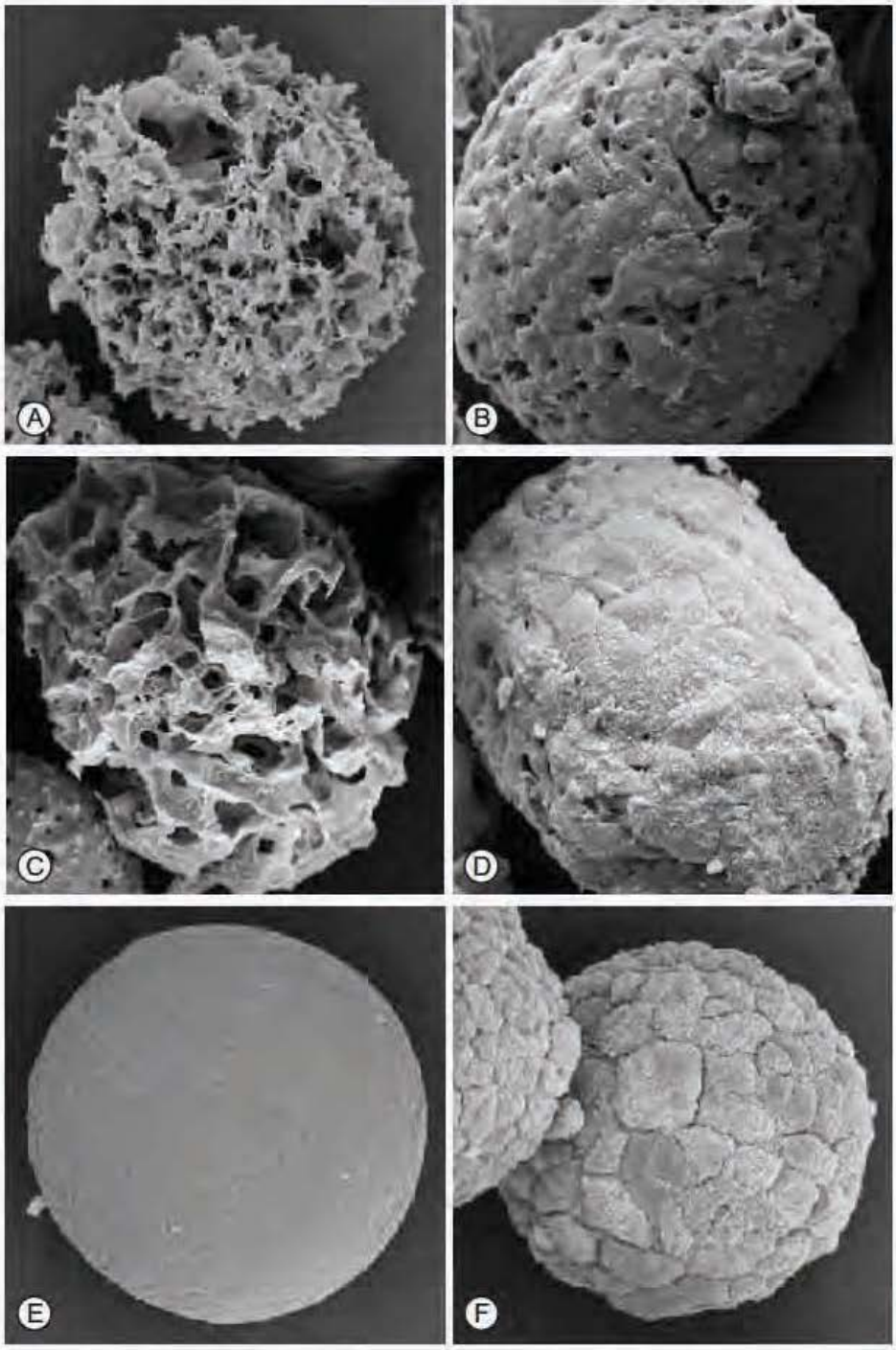


Figure 18.6

Scanning electron microscopy of Vero cells on microcarriers (Yokomizo *et al.*, 2004). (A) Preparation of a *Cytopore*™ microcarrier without cells (original magnification ×242). (B) Preparation of a cell loaded *Cytopore*™ microcarrier at day 6 (original magnification ×330). (C) Preparation of a *CultiSpher*® *G* microcarrier without cells (original magnification ×370). (D) Preparation of a cell loaded *CultiSpher*® *G* microcarrier at day 6 (original magnification ×295). (E) Preparation of a *Cytodex*™ *1* microcarrier without cells (original magnification ×520). (F) Preparation of a cell loaded *Cytodex*™ *1* microcarrier at day 6 (original magnification ×485).

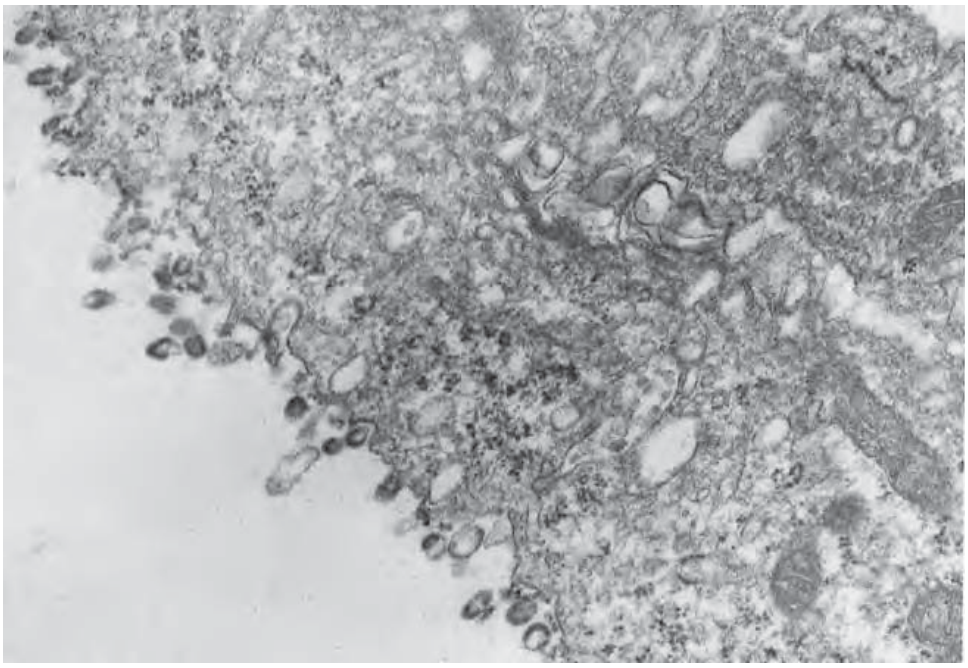


Figure 18.7

Transmission electron microscopy of Vero cell growth on a microcarrier surface, 7 days after rabies virus infection.

potential loss of productivity. Therefore, it is not only important to fully optimize cell growth and the viral infection phase to guarantee a high product concentration within the synthesis phase, but also to insure that a highly immunogenic or infective product is fully recovered in the concentration and purifying process. The most relevant optimization parameters are the multiplicity of infection (MOI) which refers to the virus amount used in the infection, and the time of infection (TOI), which refers to the point of infection in relation to the cell multiplication period. MOI and TOI are parameters that can vary from one type of virus to another or even from one cell line to another, and their impact is highly significant for increasing the productivity of the system.

18.4 Strategies for the production of virus-like particles

The main objective of immunization against viruses is the prevention or modification of the disease. However, most of the existing classical vaccines are able to prevent the disease but are not so efficient in preventing infection (Sandhu, 1994; Ellis, 1999). The development of the recombinant DNA technology made possible the creation of vaccines that do not present the typical side effects of the vaccines of attenuated or inactivated viruses. Virus-like particles (VLPs) are one of the new vaccine strategies arising from recombinant DNA technology.

As mentioned above, viruses are generally composed of a number of different proteins organized in a regular three-dimensional structure together with DNA or RNA. Viral proteins are in most cases aggregated in icosahedric membranes on which certain proteins cooperate to form the

external and internal capsids. In several virus species, viral proteins are able to form three-dimensional aggregates in the absence of nucleic acids, thus creating VLPs. Similarly, the proteins that normally interact with nucleic acids can also aggregate in their absence and form core-like particles – CLPs. Therefore, in contrast to infectious viruses, VLPs consist of viral proteins aggregated in a three-dimensional structure similar to that of the native virus but not containing viral DNA or RNA within the capsid (Cruz *et al*., 2002).

18.4.1 Advantages of VLPs

The major advantage of VLPs over individual purified antigens is the more efficient antigen presentation to the immune system, since the epitopes resulting from the three-dimensional structure of the capsid will be available for recognition by the immune system. Also, many of the viruses for which immunogenic VLPs were developed are difficult to replicate *in vitro* (e.g. HBV, parvovirus B19, Norwalk calcivirus, and human papillomavirus). Finally, it should be noted that VLPs are not infectious, thus eliminating the need for inactivation and subsequent epitope modification due to the inactivation agent, as in the case of inactivated viruses (Cruz *et al*., 2002).

18.4.2 VLP production technology

Due to the availability of the viral coding sequences, it has been possible to develop methods for the production of the viral proteins using appropriate expression vectors. The fact that VLPs are multimeric structures makes the use of complex expression technologies mandatory. In addition, viruses typically infect eukaryotic cells and thus the use of such cells is required for correct viral protein production. Although this is not necessarily true for the production of a single viral protein that does not have complex post-translational modifications, such as glycosylation and phosphorylation, for production of complete VLPs, the use of complex systems cannot be avoided. This is due to the fact that the processing of viral proteins within the cells and capsid assembly, require the mediation of host proteins – the chaperones. In recent years several systems were developed with the goal of producing viral proteins in yeast, insect, and mammalian cells. The use of yeast was first demonstrated in the production of the HBV surface antigen (HBsAg) used as a vaccine against hepatitis B (Fu *et al*., 1996). The expression of this specific protein had been previously shown using *Escherichia coli*; however, the molecules did not form VLPs and their immunogenicity was low. The vaccine against hepatitis B is an important milestone in the use of VLPs as a vaccination strategy, leading to replacement of the previously existing inactivated virus vaccine.

A second milestone in the production of viral antigens was the development of the technology associated with insect cells – the baculovirus expression system. The *Autographa californica* baculovirus produces viral particles as a part of its life cycle. These particles accumulate in the polyhedrin protein matrix, which confers protection from inactivation due to environmental factors. The polyhedrin is produced in large amounts

(1 mg/10^6 cells) under the control of a very strong promoter. The use of this promoter to drive the expression of foreign proteins became the obvious next step in producing recombinant baculoviruses. The cells derived from the ovary of the caterpillar *Spodoptera frugiperda* (Sf-9 and Sf-21) are the most widely used for the production of heterologous proteins using baculoviruses (*Table 18.2*). Nevertheless, other insect cells have also been used for this purpose, including *Trichoplusia ni* (High-Five™) (Jiang *et al*., 1998; Wang *et al*., 2000).

18.4.3 VLP composition

The proteins to be included in VLPs should be those necessary to confer the desired degree of immunogenicity. As a consequence, VLPs are often composed of more than one protein. In order to use baculovirus-infected insect cells to produce VLPs it is necessary to predefine the proteins to be included, since the expression of these proteins could determine the stability (Hyatt *et al*., 1993) and location – intra- or extracellular – of the particle (French and Roy, 1990).

The antigenicity, rather than the immunogenicity, should drive the research and development of new VLP-based vaccines and thus antibody reactivity tests should be performed as early in the process as possible. For example, a porcine parvovirus vaccine is composed of a single viral protein (VP2), which represents 95% of the native virus total protein and is able do induce antibody production in immunized animals (Rueda *et al*., 1999). In contrast, the human parvovirus B19 contains the exact same proportion of VP2 in the native virus but VLPs made solely of VP2 are unable to induce neutralizing antibodies (Brown *et al*., 1991; Tsao *et al*., 1996). In this case even a VLP containing VP1 and VP2 at a ratio of 1:24, respectively, which is very similar to that of the native virus, was not

Table 18.2 Virus-like particles (VLPs) produced in baculovirus-infected insect cells

VLPs	Cell line	Reference
Human parvovirus B19	Sf 9	Bansal *et al.*, 1993
Blue tongue virus	Sf 9, Sf 21	Roy, 1990
Epizootic hemorrhagic disease	Sf 9	Le Blois *et al.*, 1991
Hepatitis B	Sf 9	Lanford *et al.*, 1989
Hepatitis C	Sf 9	Baumert *et al.* 1998
HIV	Sf 9	Cruz *et al.*, 2002
Infectious bursal disease	Sf 9, HighFive	Wang *et al.*, 2000; Hu and Bentley, 1999
Norwalk virus	Sf 9	White *et al.*, 1997
Papillomavirus	Sf 9	Kirnbauer *et al.*, 1992
Minute virus of mice	Sf 9	Hernando *et al.*, 2000
Polyomavirus	Sf 9	Montross *et al.*, 1991
Porcine parvovirus	Sf 9, Sf 21	Maranga *et al.*, 2004; Martinez *et al.*, 1992
Rotavirus	Sf 9, HighFive	Vieira *et al.*, 2005; Jiang *et al.*, 1998
SV40	Sf 9	Kosukegawa *et al.*, 1996

efficient in inducing an immune response. The final composition of the antigenic VLP included VP1 at a percentage between 25 and 40%, that is, 6–10-fold higher than in the native viral particle (Tsao *et al*., 1996). This clearly shows that there is still a need for a better understanding of the immunization phenomenon.

Therefore, to ensure antigenicity, the particle composition has to be studied and will probably lead to different baculovirus infection strategies, using a different number of proteins per virus. For instance, the blue tongue virus (BTV), an Orbivirus from the Reoviridae family, is a non-enveloped virus that contains seven different structural proteins (VP1–VP7) (Hyatt *et al*., 1993). The outer capsid consists of two proteins, VP2 and VP5, while the core is composed of the remaining five proteins, two major (VP3 and VP7) and three minor (VP1, VP4, and VP6), classified according to their abundance. The production of BTV VLPs can be performed by co-infecting the cells with two dual-gene vectors expressing VP3 + VP5 and VP2 + VP7 (Brown *et al*., 1991) or through the infection of a multigenic vector containing all four proteins (VP2, VP3, VP5, and VP7) (Martinez *et al.*, 1992). The factors related to the VLP composition, including the use of multigenic vectors, production technology, and large-scale production VLPs, have been analyzed by Maranga *et al*. (2000a).

18.4.4 VLP production processes

Hepatitis B vaccine

HBV is transmitted among humans and is manifested as a chronic infection that debilitates infected individuals and may cause severe liver damage, primary carcinoma, and ultimately death. Although in most cases the patients recover, there are large segments of the population that have chronic hepatitis B (especially in African and Asian countries) and can transmit the disease as a pandemic.

HBV is an enveloped virus whose nucleocapsid involves one single DNA molecule. Several surface antibodies have been found against the surface antigens (HBsAg) in the serum of HBV-infected individuals. The HBsAg is present in the blood of infected individuals in the form of spherical particles with a diameter of 22 nm. Since these particles are able to induce immunization in humans, a production process based on yeast cloned with the gene encoding the HBsAg monomer has been developed. However, since yeasts do not secrete the VLPs, sonication is used to extract the particles, which are subsequently purified.

The VLPs produced by yeast consist of about 100 HBsAg monomers and confer immunization when injected into humans in spite of the different glycosylation as compared with the native virus (Fu *et al*., 1996). Nevertheless, the vaccine only confers immunity after three doses and requires revaccination after 5 years.

Production of HIV-1 and porcine parvovirus VLPs

The production processes for HIV-1 and porcine parvovirus (PPV) VLPs are presented in *Figure 18.8*. In both cases, the insect cell–baculovirus

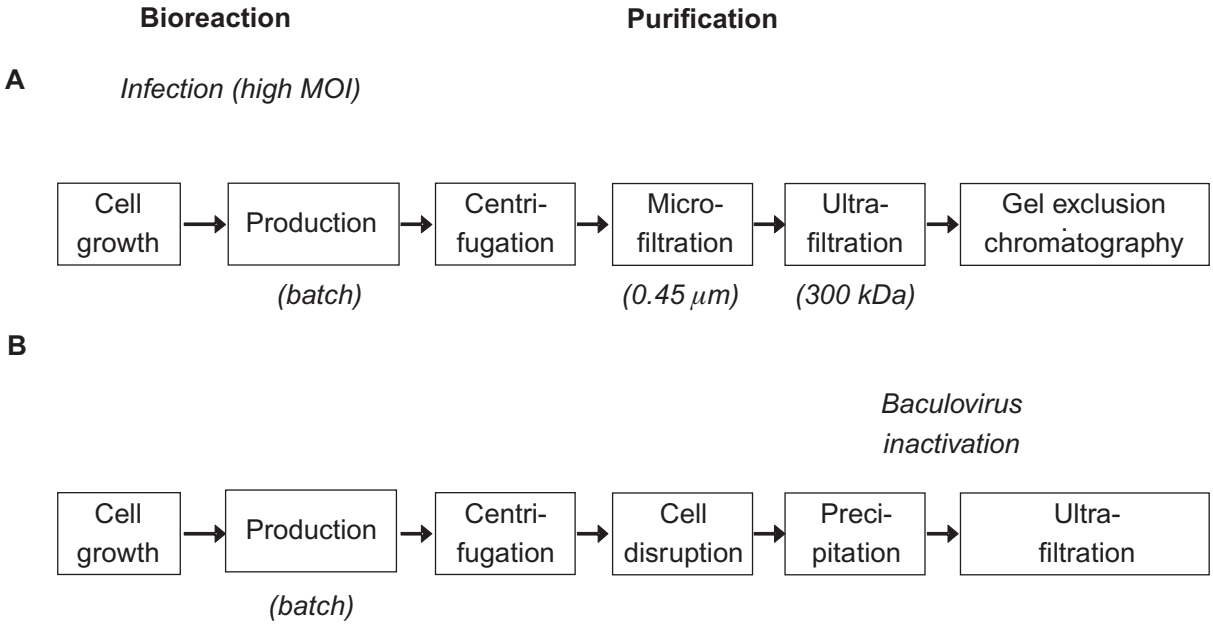


Figure 18.8

Production and downstream processing of HIV 1(A) and PPV (B) VLPs (adapted from Cruz *et al.*, 2002).

system was used but the production strategy has some significant differences. In the case of HIV-1 VLPs, Sf-9 cells were infected with a high MOI (Cruz *et al.*, 1998), while in the case of PPV VLPs, Sf-21 cells were preferred and a low MOI was used for economic reasons, as these VLPs are developed as a veterinary vaccine (Maranga *et al.*, 2004). Also, at the purification level there are some differences, since the HIV-1 VLPs are secreted and PPV VLPs are intracellular, thus requiring a cell disruption step. However, it should be noted that both downstream processing strategies are based on the relatively larger size of the VLP in comparison with other contaminants, although chromatographic steps are used only for HIV-1 VLPs (Cruz *et al.*, 2002).

18.5 Development of viruses for DNA vaccines

Free DNA-mediated immunization, also referred to as genetic immunization, consists of the administration of genetic material (DNA in the form of a plasmid), which will lead to the *in vivo* expression of proteins that induce an immune response. This vaccination method is simple and constitutes a relevant alternative to the classical vaccination, mainly due to two important advantages. First, the long-term expression of small amounts of antigens is possible, avoiding the need for re-administration, as long as no allergies, tolerance, or autoimmunity occur. Second, the antigens are synthesized *in vivo*, no infection exists and therefore inadvertent reactions are avoided and the treatment of infected individuals becomes possible. The first animal studies using this type of vaccines demonstrated their potential in the protection against the flu virus. DNA vaccines also have other advantages, namely the higher thermal, chemical,

and biological stability of DNA in comparison with classical vaccines. This lowers the requirements for storage at low temperature, thus reducing the costs, especially for developing countries.

Nevertheless, the large-scale application in healthy individuals still involves delicate safety issues, although clinical trials are expected for cancer and HIV infection.

The spectrum of target diseases can be expanded through the use of a mix of different plasmids, a strategy that is difficult to implement when using proteins due to interference. Given the ease of modification of DNA sequences, a single gene may include several epitopes of more than one antigen or different pathogenic strains.

There are several methods of administration. Free DNA can be directly injected into muscle cells (currently the most efficient method), linked to gold particles which are then bombarded onto the tissue (difficult to prepare), pulverized without injection or administered orally. In spite of the low efficiency (in terms of modified cells), this new vaccination method frequently leads to surprisingly high immune responses at both the humoral and cellular level. The technology used in this type of vaccination has the additional advantage of allowing research in the area of molecular immunology, as well as in the study of the immune system and new vaccine development due to the ease of producing plasmids encoding different proteins. This can be performed within a few weeks as compared with the months necessary for new viral vectors. Nevertheless, viral vectors, especially adenoviruses, are currently being used in DNA vaccination. The ability of adenoviruses to very efficiently induce an immune response has several advantages: (a) to provide high amounts of antigen to lymphoid tissue; (b) to induce a rapid T-cell expansion and migration in the lymphatic system; and (c) to promote and prolong T-cell response (Yang *et al*., 2003). In addition, adenoviruses are non-integrating vectors and their biology is well understood. In parallel, viral vectors based on other virus types have been developed, for example influenza virus (Ulmer *et al*., 1998). Currently, Crucell is developing two DNA vaccines against malaria and Ebola virus based on adenovirus Ad35 and Ad11, two serotypes that are not common, thus eliminating the problem of existing immunity against Ad5. In this case it is possible to obtain a good immune response and an effective protection against infection. There are several DNA vaccines under development against a number of infectious diseases (*Table 18.3*).

The application of the DNA vaccine technology is not limited to infectious diseases and is currently being used in therapeutic vaccines against cancer. *Table 18.4* presents the therapeutic DNA vaccines under development (Powell, 2004).

Further detail on gene therapy is provided in Chapter 21, which focuses on the use of viruses for therapeutic applications instead of prophylaxis.

18.6 Perspectives for the evolution of viral vaccine production

Vaccination has prevented, in a safe and efficient way, more diseases and deaths due to infectious agents than any other public health policy except

Table 18.3 DNA vaccines under development

Vector type	Target	Company	Development stage
Adenovirus	Ebola	Crucell/NIH	Preclinical
	Hepatitis C	Merck & Co.	Preclinical
	HIV	Merck & Co.	Phase I
	Malaria	Crucell/GlaxoSmithKline	Preclinical
	Rabies	Vaxin	Preclinical
	West Nile virus	Pfizer/Kimron Vet. Inst	Preclinical
Plasmids	Ebola	NIH Vaccine Research Center	Phase I
	HIV	Wyeth Lederle	Phase I complete
	HIV	Wyeth Lederle	Phase I
	HIV	Chiron	Phase I
	HIV	Epimmune	Phase I

Table 18.4 Therapeutic DNA vaccines under development

Vaccine	Company	Development stage
HIV 1	Corixa	Phase I
Melanoma	ImClone Systems	Phase I
Lung cancer	ImClone Systems	Phase III
Prostate cancer	Inovio	Phase I/II
Melanoma	M.D. Anderson Cancer Center	Phase I/II
Melanoma	Vical	Phase III

for clean water supply. Among the several international programs launched in the 20th century to eradicate diseases, the only successful one to date was based on vaccines – the eradication of smallpox. Poliomyelitis may be the next success within the next decade. *Table 18.5* shows the evolution of the viral vaccines available on the market from live/attenuated vaccines to the more recent subunit and recombinant DNA vaccines.

The major problem associated with recombinant vaccines is related to the immune response that is often only humoral (antibody production) and not cellular (e.g. cytotoxic T-lymphocytes, CTL) (Ellis, 1999). Normally, viral vaccines are able to stimulate both types of immune response, thus avoiding the need for revaccination (Ellis, 1996).

Another important issue concerns the number of vaccines used for children under 2 years of age; the vaccination program includes around 15 injections causing some discomfort not only to the children but also to their parents (Papaevangelou, 1998). It is therefore necessary to develop combined vaccines – live, inactivated or mixed with VLPs or other subunits – for which the allergic reactivity is minimal.

To circumvent these two problems and taking advantage of the VLP stability, some methods have been developed to introduce multiple epitopes in the same VLP. One example is the use of porcine parvovirus VLP containing epitopes to induce cellular immune response (B cells, CD4+, and CTL). This strategy should allow the production of cheaper and more potent vaccines that will in turn induce a more effective immune response

Table 18.5 Viral vaccines: historical perspective

Year	Disease	Vaccine type
1796	Rubeola	Live/attenuated*
1885	Rabies	Killed/inactivated*
1928	Yellow fever	Live/attenuated
1936	Flu	Killed/inactivated*
1937	Encephalitis	killed/inactivated
1945	Japanese encephalitis B	Killed/inactivated
1955	Poliomyelitis	Killed/inactivated
1958	Poliomyelitis	Oral vaccine (live/attenuated)
1963	Measles	Live/attenuated
1967	Mumps	Live/attenuated
	Rubeola	Live/attenuated
1981	Hepatitis B	Protein
1983	Varicella zoster	Live/attenuated
	HB	Protein/recombinant DNA subunit
	Flu	Killed/inactivated
	Hepatitis A	Subunit, with adjuvant
1997	Flu	Subunit, with adjuvant
2000	HA Typhoid fever	Polysaccharide
2003	Flu	Live/attenuated
	Meningococcal disease	Serotypes ACW polysaccharide
2004	Rotavirus	Live/attenuated

*Substituted by a new vaccine.

(Rueda et al., 1999; Maranga *et al.*, 2000b). This type of chimera is currently under development using HBV VLPs, yeast Ty particles, and blue tongue virus VLPs, as well as other polioviruses.

A good example of the recent and future evolution of viral vaccines and their concomitant issues of technology, complexity and competition, is the rotavirus vaccine. This is of great relevance for the prevention of diarrhea, which is often deadly in developing countries (half a million deaths per year) and has high hospitalization costs in rich countries. After successive failures of monovalent vaccines, multivalent vaccines based on the reshuffling of rotavirus strains comprising the attenuation properties of animal strains with the external capsid of human serotypes were developed.

The first vaccine (Rotashield®, developed by Wyeth Lederle) entered the market in August 1998, after extensive clinical trials providing efficient protection (> 90%) and safety. After less than 1 year 15 cases of intussusception were reported, leading to the withdrawal of this vaccine from the market (Murphy *et al.*, 2003, Fischer *et al.*, 2004). Because this risk was calculated as being lower than 1 in 5000 cases, the two major candidate vaccines (pentavalent human-bovine reshuffling, Rotateq® Merck & Co. and monovalent human attenuated Rotarix®, Glaxo Smith Kline) are currently under the most extensive Phase III clinical trial record – over 60 000 patients each! This fact can give an idea of the increasing standard of what is considered to be an acceptable risk in vaccination programs (with the obvious exception of anticancer vaccines in initial stages and therefore without clearly defined acceptance standards). This shows an evident fact in vaccine praxis: the ''easy'' vaccines have already been

introduced in the market and the ''difficult'' vaccines, namely viral vaccines, are the only ones left.

For this reason, there are several groups in Europe (involving the authors of this chapter) and in the USA looking for VLPs produced in insect cells using multicystronic baculovirus presenting different complementary DNA encoding the critical viral proteins VP2, VP6, VP7, and, eventually, VP4/VP8 in the same vector under the control of different promotors (Vieira *et al*., 2005). Using this technology, after validation of the humoral and cellular response, it is possible to consider systems biology strategies to understand the replication, stability, mRNA kinetics, protein production stoichiometry, and VLP packaging. Such knowledge will allow the improvement of the molecular biology of the baculovirus vector and the number of proteins generating appropriate VLPs.

The use of DNA vaccines could also bring additional benefits since they are based on technological platforms (such as the adenoviral platform AdVac from Crucell), thus providing great flexibility. It is possible in these cases to add new antigens (derived from a virus, parasite of bacteria) to obtain a greater efficacy through the modulation of the immune response to the mechanism of action of each pathogen. This type of technology is particularly useful in cases where the production of antigen is not only difficult but also dangerous (HIV-1, Ebola virus, and West Nile virus). Nevertheless, there are still important challenges, such as the definition of formulations that significantly increase the stability of the vaccine.

It is therefore expected that subunit vaccines will proliferate in the market because they are predominantly safe, as long as their evolution is supported by the development of new technologies that ensure efficacy.

References

Bansal GP, Hatfield JA, Dunn FE, Kramer AA, Brady F, Riggin CH, Collett MS, Yoshimoto K, Kajigaya S, Young NS (1993), Candidate recombinant vaccine for human B19 parvovirus, J. Infect. Dis. 167:1034 1044.

Baumert TF, Ito S, Wong DT, Liang TJ (1998), Hepatitis C virus structural proteins assemble into virus like particles in insect cells, J. Virol. 72:3827 3836.

Brown CS, Van Lent JW, Vlak JM, Spaan WJ (1991), Assembly of empty capsids by using baculovirus recombinants expressing human parvovirus B19 structural proteins, J. Virol. 65:2702 2706.

Butler M (1987), Growth limitations in microcarrier cultures, Adv. Biochem. Eng. Biotechnol. 34:57 84.

Butler M, Spier RE (1984), The effects of glutamine utilization and ammonia produc tion on the growth of BHK cells in microcarrier cultures, J. Biotechnol. 1: 187 196.

Croughan MS, Hamel JF, Wang DIC (1987), Hydrodynamic effects on animal cells grown in microcarrier cultures, Biotechnol. Bioeng. 23:130 141.

Cruz PE, Cunha A, Peixoto CC, Clemente J, Moreira JL, Carrondo MJT (1998), Optimization of the production of virus like particles in insect cells, Biotechnol. Bioeng. 60:408 418.

Cruz PE, Maranga L, Carrondo MJT (2002), Integrated process optimization: lessons from retrovirus and virus like particle production, J. Biotechnol. 99:199 214.

Ellis RW (1996), The new generation of recombinant viral subunit vaccines, Curr. Opin. Biotechnol. 7:646 652.
Ellis RW (1999), New technologies for making vaccines, Vaccine 17:1596 1604.
Fischer TK, Bresee JS, Glass RI (2004), Rotavirus vaccines and the prevention of hospital acquired diarrhea in children, Vaccine 22(Suppl 1):S49 54.
Frazzatti Gallina NM, Paoli RL, Mourão Fuches RM, Jorge SAC, Pereira CA (2001), Higher production of rabies virus in serum free medium cell cultures on micro carriers, J. Biotechnol. 92:67 72.
French TJ, Roy P (1990), Synthesis of bluetongue virus (BTV) core like particles by a recombinant baculovirus expressing the two major structural core proteins of BTV, J. Virol. 64:1530 1536.
Fu J, VanDusen W, Kolodin DG, O'Keefe DO, Herber WK, George HA (1996), Continuous culture study of the expression of hepatitis B surface antigen and its self assembly into virus like particles in *Saccharomyces cerevisiae*, Biotechnol. Bioeng. 49:578 586.
Griffiths JB, Cameron DR, Looby D (1987), A comparison of units process systems for anchorage dependent cells, Dev. Biol. Stand. 66:331 330.
Hernando E, Llamas Saiz AL, Foces Foces C, McKenna R, Portman I, Agbandje McKenna M, Almendral JM (2000), Biochemical and physical characterization of parvovirus minute virus of mice virus like particles, Virology 267:299 309.
Hu YC, Bentley WE (1999), Enhancing yield of infectious Bursal disease virus structural proteins in baculovirus expression systems: focus on media, protease inhibitors, and dissolved oxygen, Biotechnol Prog. 15:1065 1071.
Hu WS, Wang DIC (1987), Selection of microcarrier diameter for the cultivation of mammalian cells on microcarriers, Biotechnol. Bioeng. 30:548 557.
Hyatt AD, Zhao Y, Roy P (1993), Release of bluetongue virus like particles from insect cells is mediated by BTV nonstructural protein NS3/NS3A, Virology 193:592 603.
Jiang B, Barniak V, Smith RP, Sharma R, Corsaro B, Hu B, Madore HP (1998), Synthesis of rotavirus like particles in insect cells: comparative and quantitative analysis, Biotechnol. Bioeng. 60:369 374.
Kent BL, Mutharasani R (1992), Cultivation of animal cells in a reticulated vitreous carbon foam, J. Biotechnol. 22:311 328.
Kirnbauer R, Booy F, Cheng N, Lowy DR, Schiller JT (1992), Papillomavirus L1 major capsid protein self assembles into virus like particles that are highly immunogenic, Proc. Natl Acad. Sci. U S A 89:12180 12184.
Kosukegawa A, Arisaka F, Takayama M, Yajima H, Kaidow A, Handa H (1996), Purification and characterization of virus like particles and pentamers produced by the expression of SV40 capsid proteins in insect cells, Biochim. Biophys. Acta 1290:37 45.
Lanford RE, Luckow V, Kennedy RC, Dreesman GR, Notvall L, Summers MD (1989), Expression and characterization of hepatitis B virus surface antigen polypeptides in insect cells with a baculovirus expression system, J. Virol. 63:1549 1557.
Le Blois H, Fayard B, Urakawa T, Roy P (1991), Synthesis and characterization of chimeric particles between epizootic hemorrhagic disease virus and bluetongue virus: functional domains are conserved on the VP3 protein, J. Virol. 65: 4821 4831.
Maranga L, Cruz PE, Aunins JG, Carrondo MJT (2002a), Production of core and virus like particles with baculovirus infected insect cells, Adv. Biochem. Eng. Biotechnol. 74:183 206.
Maranga L, Rueda P, Antonis AF, Vela C, Langeveld JP, Casal JI, Carrondo MJT (2002b), Large scale production and downstream processing of a recombinant porcine parvovirus vaccine, Appl. Microbiol. Biotechnol. 59:45 50.

Maranga L, Cunha A, Clemente J, Cruz PE, Carrondo MJT (2004), Scale up of virus like particles production: effects of sparging, agitation and bioreactor scale on cell growth, infection kinetics and productivity, J. Biotechnol. 107:55 64.
Martinez C, Dalsgaard K, Lopez de Turiso JA, Cortes E, Vela C, Casal JI (1992), Production of porcine parvovirus empty capsids with high immunogenic activity, Vaccine 10:684 690.
Mendonça RZ, Ioshimoto LM, Mendonça RMZ, De Franco M, Valentini EJG, Beçak W, Pereira CA (1993), Preparation of human rabies vaccine in VERO cell culture using a microcarrier system, Braz. J. Med. Biol. Res. 26:1305 1317.
Mendonça RZ, Prado JCM, Pereira CA (1999), Attachment, spreading and growth of VERO cells on microcarriers for the optimization of large scale cultures, Biopro cess Eng. 20:565 571.
Mendonça RZ, Arrózio SJ, Antoniazzi MM, Ferreira JMC Jr, Pereira CA (2001), Metabolic active high density VERO cell cultures on microcarriers following apoptosis prevention by galactose/glutamine feeding, J. Biotechnol. 97:13 22.
Montagnon B, Vincent Falquet JC, Fanget B (1984), Thousand litre scale microcarrier culture of VERO cells, Dev. Biol. Stand. 55:37 42.
Montross L, Watkins S, Moreland RB, Mamon H, Caspar DL, Garcea RL (1991), Nuclear assembly of polyomavirus capsids in insect cells expressing the major capsid protein VP1, J. Virol. 65:4991 4998.
Murphy TV, Smith PJ, Gargiullo PM, Schwartz B (2003), The first rotavirus vaccine and intussusception: epidemiological studies and policy decisions, J. Infect. Dis. 187:1309 1313.
Nahapetian AT, Thomas JN, Thilly WG (1986), Optimization of environment for high density VERO cell culture: effect of dissolved oxygen and nutrient supply on cell growth and changes in metabolites, J. Cell Sci. 81:65 103.
Papaevangelou G (1998), Current combined vaccines with hepatitis B, Vaccine 16: S69 72.
Powell K (2004) DNA vaccines back in the saddle again?, Nat. Biotechnol. 22: 799 801.
Reuveny S, Coret R, Freeman A, Kotler M (1985), Newly developed microcarrier culturing systems an overview, Dev. Biol. Stand. 60:243 253.
Roy P (1990), Use of baculovirus expression vectors: development of diagnostic reagents, vaccines and morphological counterparts of bluetongue virus, FEMS Microbiol. Immunol. 2:223 234.
Rueda P, Martinez Torrecuadrada JL, Sarraseca J, Sedlik C, del Barrio M, Hurtado A, Leclerc C, Casal JI (1999), Engineering parvovirus like particles for the induction of B cell, CD4(+) and CTL responses, Vaccine 18:325 332.
Sandhu JS (1994), Engineered human vaccines, Crit. Rev. Biotechnol., 14:1 27.
Tsao EI, Mason MR, Cacciuttolo MA, Bowen SH, Folena Wasserman G (1996), Production of parvovirus B19 vaccine in insect cells co infected with double baculoviruses, Biotechnol. Bioeng. 49:130 138.
Ulmer JB, Fu TM, Deck RR, Friedman A, Guan L, DeWitt C, Liu X, Wang S, Liu MA, Donnelly JJ, Caulfield MJ (1998), Protective CD4+ and CD8+ T cells against influenza virus induced by vaccination with nucleoprotein DNA, J. Virol. 72:5648 5653.
Van Wezel AL (1967), Growth of strains and primary cells on microcarriers in homogeneous culture, Nature 216:64 65.
Varani J, Dame M, Beals TF, Wass JA (1983), Growth of three established cell lines on glass microcarriers, Biotechnol. Bioeng. 25:1359 1372.
Vieira HLA, Estevão C, Roldão A, Peixoto C, Sousa M, Cruz PE, Carrondo MJT, Alves PM (2005), Triple layered rotavirus VLP assembly: kinetics of vector replication, mRNA stability and recombinant protein production, J. Biotechnol. 120:72 82.

Wang MY, Kuo YY, Lee MS, Doong SR, Ho JY, Lee LH (2000), Self assembly of the infectious bursal disease virus capsid protein, rVP2, expressed in insect cells and purification of immunogenic chimeric rVP2H particles by immobilized metal ion affinity chromatography, Biotechnol. Bioeng. 67:104 111.

White LJ, Hardy ME, Estes MK (1997), Biochemical characterization of a smaller form of recombinant Norwalk virus capsids assembled in insect cells, J. Virol. 71: 8066 8072.

Yang TC, Dayball K, Wan YH, Bramson J (2003), Detailed analysis of the CD8+ T cell response following adenovirus vaccination, J. Virol. 77:13407 13411.

Yokomizo AY, Antoniazzi MM, Galdino PL, Azambuja NJ, Jorge SAC, Pereira CA (2004), Rabies virus production in high Vero cell density cultures on macroporous microcarriers, Biotechnol. Bioeng. 85:506 515.

Bioinsecticides

19

Márcia Regina da Silva Pedrini and
Ronaldo Zucatelli Mendonça

19.1 Introduction

This chapter describes the application of animal cells, particularly insect cells, for baculovirus production and its use as a bioinsecticide. The use of baculovirus in the control of insect pests and its production *in vitro* are discussed.

Brazil is one of the largest agricultural producers in the world. According to data from the Institute of Agriculture (IEA), in 2003 the economic balance of the Brazilian agro business was US$ 17.61 billion (Vicente *et al*., 2003). In 2005, the Brazilian production was about 113 million tons of grains, with soy accounting for about 48% of this production (IBGE, 2005).

Pesticides have an important role in the development of agricultural production, in controlling pests and insuring a sustainable agriculture. However, the constant use of chemical pesticides, in an indiscriminate way, causes a reduction of the population of beneficial organisms and induces resistance of the targeted insect pests, which creates even more dependence on chemical products. Therefore, the reduction of the usage of these pesticides in agriculture is of great importance for commercial and environmental reasons.

The biological control of insect pests in agriculture is an old practice. More than 120 years ago, an infestation of citrus fruit production by *Icerya purchasi* in Florida State (USA) was treated using a ladybug from Australia, a procedure that was later used in other countries, including Brazil (Carvalho *et al*., 1999). Currently, several bioinsecticides are used for biological control, the most common being insects (wasps, acarids, etc.), bacteria (*Bacillus thuringiensis*), fungi, or virus (baculovirus).

Bioinsecticides based on baculovirus have had a strong impact on the production of grain. Brazilian research on the use of baculovirus was initiated by EMBRAPA (Brazilian Agricultural Research Corporation) during the 1970s (Moscardi, 1993). Because of this research, currently, soy farming is maintained entirely without the use of chemical pesticides. The biologically treated area exceeds two million hectares, distributed among several different states of Brazil. This is the best worldwide example of large-scale viral biopesticide usage (Agência Brasil, 2004).

In such a system, a species-specific virus is used. In the case of soy cultivation, this virus is *Baculovirus anticarsia*, which specifically kills a caterpillar found on the soy – *Anticarsia gemmatalis* – a defoliating pest that substantially reduces farming productivity. With this bioinsecticide,

the use of millions of liters of chemical insecticide per harvest is avoided, thus generating an enormous environmental and economical benefit to the country (EMBRAPA, 2002).

In addition to the economic and environmental gains, other advantages of this product are the facts that the manipulation of chemicals can be avoided and that the virus is species-specific. It only affects invertebrates and causes no harm to other insects that could be natural bioinsecticides to other pests. Moreover, other factors that contribute to its success are: the high virulence of the virus and its infection efficiency, the low cost of the product compared with chemical insecticides, and the fact that this is a unique pest in most producing areas. The sum of these advantages makes the baculovirus very attractive as a bioinsecticide (Moscardi, 1999; Moscardi and Souza, 2002; Szewczyk *et al*., 2006).

Nevertheless, the bioinsecticide has some limitations. Although the high specificity is very important in ecological terms, this restricts its market. The bioinsecticide has low activity. However, this can be improved through modification of the baculovirus genome, suppressing or incorporating genes from other organisms, such as those that express hormones or toxins. According to Szewczyk *et al*. (2006), genetically modified baculovirus will be gradually introduced into countries that have a general acceptance of genetically modified organisms (GMOs).

Another aspect to be considered is the need for increased production. At the moment, the production of the bioinsecticide is accomplished by harvesting infected caterpillars in the areas infected or by growing the caterpillars in a laboratory. In the first case, there is great variability in productivity from year to year, since the production depends on the insect abundance during each harvest, which varies with multiple factors. However, there has been considerable progress in the production of the virus under laboratory-controlled conditions (Moscardi and Santos, 2005). In March of 2005 EMBRAPA finished the construction of a pilot plant with the capacity to inoculate about 30 000 caterpillars per day, and another institution, the COODETEC (Cascavel, PR), is enlarging its production capacity to 600 000 caterpillars per day. However, the caterpillars have to be fed with artificial diets and the cost of production of a dose of the biopesticide, based on *Baculovirus anticarsia*, using raw material produced locally, is approximately 90% higher than that obtained by direct harvesting from the field. The use of alternative formulations, such as substituting agar for carrageen as a gelling agent, has reduced production cost, making it more economically feasible to produce the bioinsecticide in the laboratory (Santos, 2003). However, this type of process has high labor demands. Large-scale virus production in cell culture in bioreactors is a desirable development, since it allows virus multiplication in a smaller area and it reduces labor requirement, in comparison with *in vivo* production in the laboratory.

19.2 Baculovirus as a bioinsecticide: mechanism of action

Baculovirus belongs to the Baculoviridae family, which has two different genera: Nucleopolyhedrovirus (NPV) and Granulovirus (GV). Baculo-

virus exists as two phenotypes designated extracellular virus or budded virus (BV) and the occlusion bodies, also known as OBs, COPs, GDP, or polyhedra (*Figure 19.1*). The occlusion bodies are structures that are formed inside the nucleus of the infected insect cells by baculovirus (Friesen and Miller, 2001). These are highly resistant and able to stay viable in the environment for several years under different climatic conditions. The protected viral particles (virions) are known as ODVs (occlusion-derived virus) (Harrap, 1972).

The protein contained in the GV OBs is called granulin and that in the NPV is called polyhedrin (Rohrmann, 1999; Winstanley and O'Reilly, 1999). GVs are smaller than NPVs and possess rounded OBs with just one virion occluded (Winstanley and O'Reilly, 1999). NPVs present a polyhedral form and they can be multiple type (MNPV) (*Figure 19.1A*) or simple type (SNPV) (*Figure 19.1B*), depending on the number of capsids per virion (Rohrmann, 1999). Since most of the baculovirus used as biopesticidas are NPVs, in this chapter the OBs will be referred as polyhedra.

The names of different baculovirus species come from the host name in which the virus was initially identified in nature. In this way, each baculovirus species receives the name of this host and the name of the family to which the virus belongs.

The infection cycle is shown in *Figure 19.2*. When the polyhedra are dispersed in the environment, the viral particles inside them (ODVs) are deposited on the plant leaves. When the caterpillars feed on the virus-contaminated foliage, they ingest the polyhedra. The alkaline environment in the caterpillar medium intestine causes breakdown of the polyhedra and the viral particles are released from the polyhedra. The infection of the cells occurs via a receptor-mediated fusion process. Once in the cytoplasm, the nucleocapsids without membrane are transported to the nucleus of the cell, where gene expression and genome replication begins.

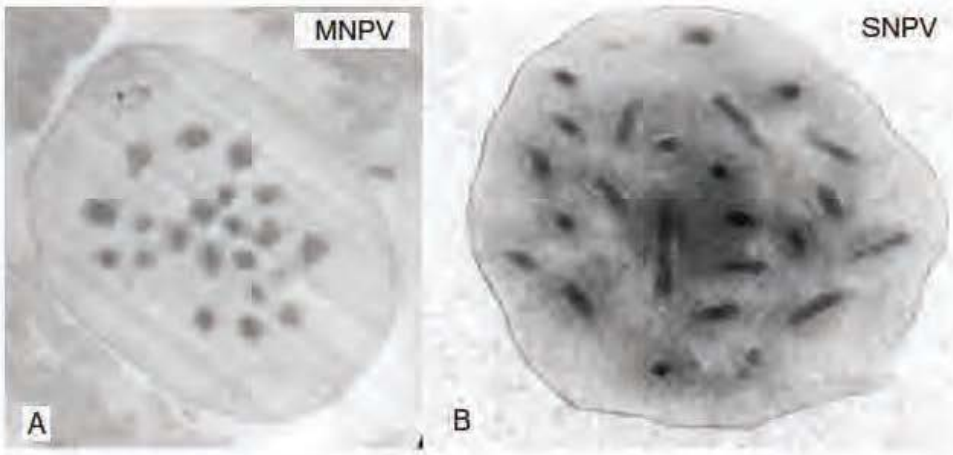


Figure 19.1

Electron micrographs illustrating polyhedra produced by nucleopolyhedrovirus in insect cell culture with multiple nucleocapsids per envelope (A), or with single nucleocapsids per envelope (B).

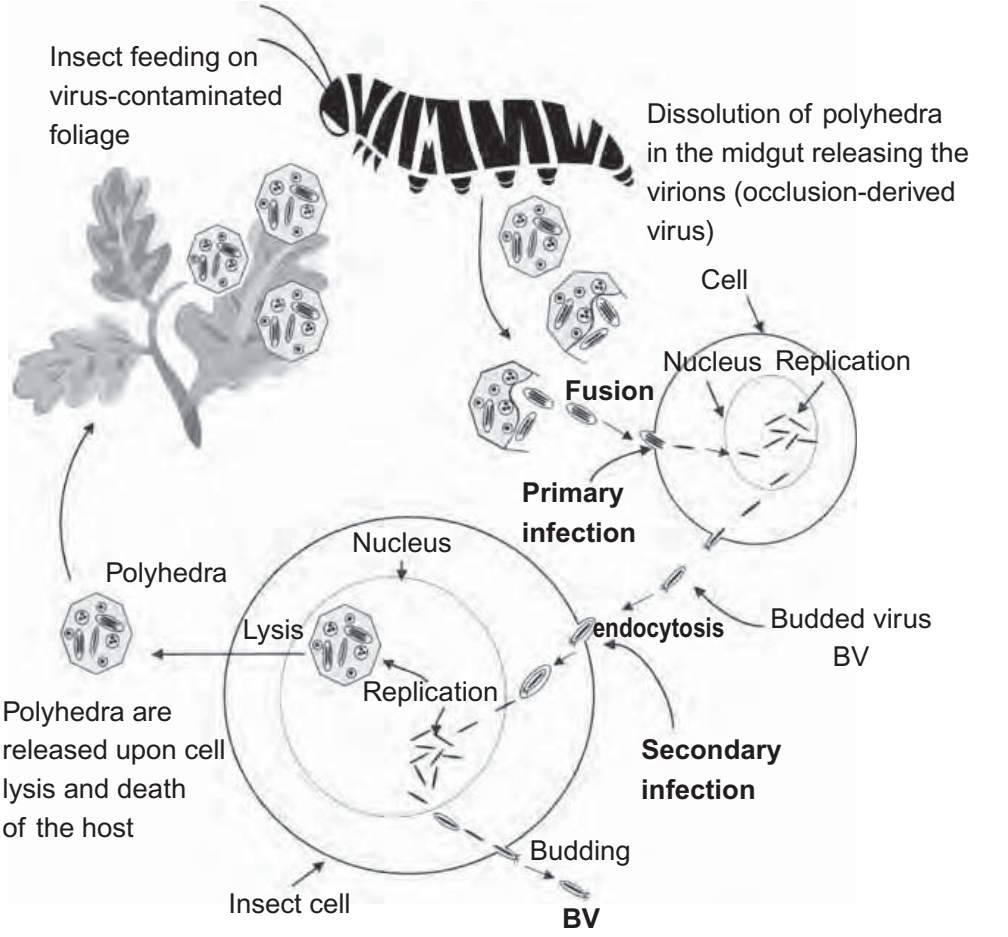


Figure 19.2

Diagram of the life cycle of a nucleopolyhedrovirus.

The nucleocapsids are envelopes that contain a nucleoprotein in the center, which transports the viral DNA to the cell nucleus. After replication, new copies of the viral genome are encapsulated in the nucleus, creating new nucleocapsids. The nucleocapsids then migrate to the cytoplasm and leave the cell by a budding process. In this process, the nucleocapsids become enveloped by the acquisition of the host cell plasma membrane. The resulting virions are extracellular (budded virus, BV) and these constitute the main products of the first stage of the infection process in the caterpillar, known as primary infection.

The BVs are taken to other tissues of the larva by the tracheal system, where they enter the cells by endocytosis, starting with the secondary infection process in which the infection is spread to all cells of the host organism. Two virion types are produced in this stage of the infection. Some of the newly synthesized nucleocapsids migrate to the cytoplasm and are released from the cytoplasmic membrane, forming new BVs. Other nucleocapsids are enveloped again in the nucleus. The virions that are occluded (ODVs), are surrounded by the protein crystal structure, forming the polyhedra. In the final phase of infection, the larva becomes cream in color and the outer covering (tegument) becomes fragile. The

rupture of the tegument causes the release of the polyhedra, which are then available for the infection of other caterpillars (Federici, 1986; Granados and Federici, 1986; Adams and Bonami, 1991; Rohrmann, 1999; Winstanley and O'Reilly, 1999; Friesen and Miller, 2001).

19.3 Animal cell cultures for baculovirus production

The insect baculovirus–cell system has been widely used, mainly for the production of recombinant proteins (Maiorella *et al*., 1988; Jarvis, 1997). This system has several advantages, including the ability to produce functional recombinant proteins that are immunogenically active, the ability to make post-translation modifications, and the fact that it contains a powerful promoter (polyhedrin), as well as the fact that the virus is not pathogenic to plants and vertebrates (Caron *et al*., 1990; Nguyen *et al*., 1993; Godwin *et al*., 1996).

However, for bioinsecticide production there are specific factors to be taken into account:

(i) The large-scale cell production to be used in virus production, since large volumes of cells are necessary, at a competitive cost.
(ii) Economic production processes and cheap culture media are needed to make production viable.
(iii) It is necessary to establish the most effective cell line for viral production with high virus per cell productivity. The productivity depends on both the cell type and virus.
(iv) Mutant generation must be monitored and avoided. The risk increases with the number of passages of the virus in cells.
(v) It is necessary to maintain viral virulence when cultured *in vitro*. It has been shown that there is a tendency for a loss of virulence with viral passage in cell culture.
(vi) The activity of the polyhedra produced *in vitro* should be compared with those obtained in caterpillars.

19.4 Effect of culture medium, cell line, and virus isolate on biopesticide production

Lepidoptera-derived insect cell lines are used for biopesticide production. Sf21 cells, originated from pupal ovarian tissue of the fall armyworm *Spodoptera frugiperda*, and Sf9 cells, a subclone of the Sf21 cells, are the most widely used cell lines for biopesticide production (King and Possee, 1992) (*Figure 19.3*). The availability of insect cell lines from Invitrogen (La Jolla, EUA) was an important step for increasing the use of these cell lines worldwide.

The establishment of insect cell line cultures allowed a detailed study of the infection cycle of many baculoviruses. These cell lines are easily cultured *in vitro* and their maintenance is relatively simple. The majority of the cell lines can be cultivated using a temperature of 25–30°C. However, the best temperature for the growth and infection of Sf9 cells is around 27–28°C (King and Possee, 1992).

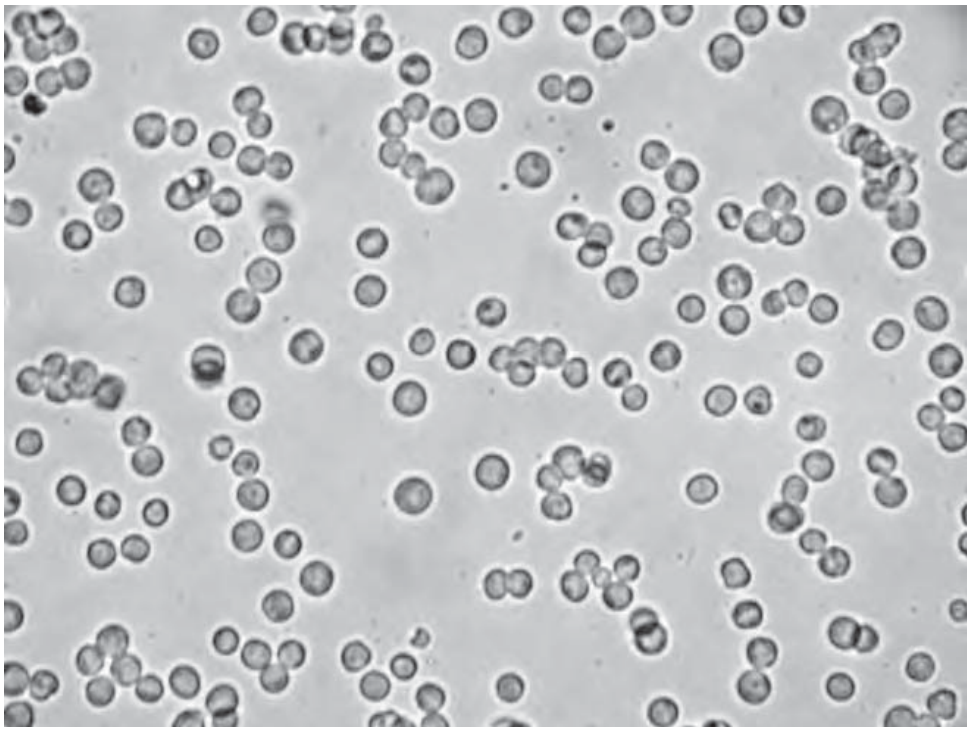


Figure 19.3

Contrast micrograph of Sf9 cells.

An important aspect of biopesticide production using insect cell lines is the maximization of cell growth. Culture media must satisfy the environmental and nutritional requirements of the cell line. Hence, many studies have been carried out to characterize the nutrient requirements and metabolic pathways of these cells (see Chapters 4 and 5).

Polyhedra bioactivity can be compromised due to nutrient limitations, causing abnormalities in virion development and occlusion within polyhedra (Rollinson *et al*., 1965; Slavicek *et al*., 1995; Belloncik *et al*., 1997). The most important aspects of culture medium selection are pH, osmolarity, and organic salt components. Components such as amino acids, vitamins, and carbon source (glucose for example), are typically found in the basal culture medium formulation. However, these simple formulations cannot promote cell growth themselves, unless supplemented with animal serum, normally 5–20% (v/v) fetal bovine serum (FBS) (Schlaeger, 1996).

Lynn (1999) demonstrated that cell maintenance for long periods in serum-free medium can lead to a slight decline in viral production. However, Chakraborty *et al*. (1999) obtained lower polyhedra production when medium with FBS was used. Therefore, medium choice is not trivial. Even though FBS has great importance for cell growth, its usage has many disadvantages. It is expensive, so its addition to the culture medium increases total costs substantially. Moreover, its quality can vary from batch to batch, with an alteration of composition, which can affect cell growth and virus replication (Schlaeger, 1996).

Development of serum-free medium has great value for large-scale biopesticide production. The latest formulations are serum-free, such as SF900II (GIBCO®/Invitrogen) and EX-CELL™ (JRH Biosciences). Cell culture medium supplementation using yeast extract (usually from alcoholic fermentation processes), milk, or soy protein concentrates, can also be an alternative to decrease cell culture medium costs (more details can be found in Chapter 5).

In vitro baculovirus production can also be affected by the cell line. Polyhedra production yield can vary between different tissues within the same host as well as in different cell lines. Furthermore, the cell line itself may be a source of viral instability in the form of transposon-mediated mutagenesis (Fraser *et al*., 1985). (Transposons are sequences of DNA that can move around to different positions within the genome, a process called ''transposition.'' In the process, they can cause mutations, and change the amount of DNA in the genome.)

Insect cell lines present different susceptibilities to virus replication. A cell line is called permissive when it can support full virus replication and all stages of viral cycle are completed, leading to polyhedra production and lysis of the host cell. Semi-permissive cell lines allow partial virus replication, possibly due to a restriction at a particular viral cycle stage (Bilimoria *et al*., 1992). Abortive cell lines are those in which the resulting virus may have a cytopathic effect, but there are no infective particles produced (Carpenter and Bilimoria, 1983; Liu and Bilimoria, 1990).

In vitro infection can be established routinely using infected larvae hemolymph containing viral infectious particles (BVs). These particles can be used to inoculate susceptible cells (see secondary infection, *Figure 19.2*). NPV replication in insect cell lines seems to be more efficient than GV replication *in vitro*. While many studies have demonstrated the susceptibility of NPVs to different cell lines, few efficient replication systems have been obtained for GVs (Miltenburger *et al*., 1984; Winstanley and Crook, 1993).

In a study carried out by Castro *et al*. (1997), the authors evaluated the susceptibility of four different lepidopteran cell lines to AgMNPV virus. *Anticarsia gemmatalis* (UFL-AG-286) and *Spodoptera frugiperda* (IPLBSF-21) infected cells were productive, whereas *Choristoneura fumiferana* (IPRI-CF-124T) and *Bombyx mori* (BM-5) infected cells were abortive.

In addition, even for permissive cells, virus yield can differ between cell lines. In the same study (Castro *et al*, 1997), UFL-AG-286 cells were shown to be more efficient than IPLBSF-21 cells in producing virus. Lynn (2003b) tested 12 insect cell lines for susceptibility to infection by three different NPVs. The author observed that virus titer differed up to 10^4-fold between cell lines. Miltenburger *et al*. (1984) established more than 200 *Cydia pomonella* primary cell lines. Of these, 81 were selected for replication of two groups of baculovirus: *Choristoneura murinana* NPV and *Cydia pomonella* GV. Although the selected cell lines were from the same insect species, a large difference in viral infection was noted, leading to different polyhedra yields. Goodman *et al.* (2001) and Lynn (2003b) also found substantial differences in susceptibility to baculovirus infection

between cell lines of the same insect species, probably due to the kind of tissues involved.

Other examples of *in vitro* baculovirus systems (virus–cell line) are: *Helicoverpa armigera* SNPV and *Helicoverpa zea* cells, with 222 polyhedra per cell (Lua et al., 2002); *Trichoplusia ni* NPV and *Trichoplusia ni* cells, with 70 polyhedra per cell (Potter et al., 1976); *Lymantria dispar* MNPV and *Lymantria dispar* cells, with 57 polyhedra per cell (Slavicek *et al*., 1996); *Spodoptera frugiperda* MNPV and *Spodoptera frugiperda*, with 399 polyhedra per cell (Almeida *et al*., 2005).

19.5 Polyhedra virulence and characteristics

Cell lines inoculated with OBs are not infectious because the virions are not released into the neutral pH of the culture medium and thus not available for further infection (Elam *et al*., 1990). In contrast, it is relatively easy to establish continuous cultures of virus isolates *in vitro* with nonoccluded virions (BVs) from infected insect hemolymph or from the supernatants of previously infected cell lines (King and Possee, 1992). In a comparative study, Volkman *et al.* (1976) estimated that BV is about 1700–1900-fold more infectious than ODV. Therefore, the use of BV is usually the first choice to propagate a virus in cell culture (King and Possee, 1992).

It is possible to use ODVs as a source of infectious virions if they are released from the OBs using alkaline solutions. But when OBs are dissolved in alkaline solution before infection of cell lines, there is only a low level of *in vitro* activity (Vail *et al*., 1979; Elam *et al*., 1990). However, this process can be improved by using proteinases. McIntosh *et al*. (1988) achieved an improvement in the insect cell culture susceptibility to ODVs using a treatment with proteinase K to dissolve *Helicoverpa zea* NPV (HzSNPV) polyhedra, which could infect homologous cells. Lynn (1994) also achieved the same result when he used a treatment of dissolving *Lymantria dispar* NPV (LdMNPV) polyhedra with trypsin.

How proteinase works to achieve this improvement has not yet been elucidated. Lynn (2003a) demonstrated that, in spite of the fact that trypsin treatment can increase infectivity of baculovirus, this enhancement does not happen for all cell–virus systems. AcMNPV infectivity of *Trichoplusia ni* (TN-368) cells increased 4750-fold using trypsin. For *Anagrapha falcifera* NPV (AfMNPV), the improvement was 77 000- fold. On the other hand, the infectivity of these two viruses for the *Lymantria dispar* (LIPLB-LdE) cell line was slightly reduced.

Some studies have demonstrated that the source of polyhedra does not affect its lethality or the time necessary to kill the caterpillars (Faulkner and Henderson, 1972; Ignoffo *et al*., 1974; Wang *et al*., 1992; Chakraborty *et al*., 1999). However, Bonning *et al*. (1995) found that polyhedra produced from caterpillars kill *Trichoplusia ni* and *Heliothis virescens* caterpillars faster than polyhedra from cell culture. This could suggest efficiency differences of these polyhedra produced from different sources. Nevertheless, there were no observed differences in lethal dose between the polyhedra tested.

Morphological analysis showed different sizes for polyhedra produced from cell culture and caterpillar (Bonning *et al*., 1995). On other hand, the polyhedra number is inversely proportional to cell size (Federici, 1986). In addition, the polyhedra produced *in vitro* is more sensitive to detergent (sodium dodecyl sulfate, SDS) treatment than polyhedra produced *in vivo* (Lua *et al*., 2003). All these differences may be related to biochemical differences observed between the infected cells *in vitro* and *in vivo* (Bonning *et al*., 1995). Another characteristic of polyhedra produced *in vitro* is the so-called ''passage effect'', which is discussed below.

19.6 Production of viral mutants in cell culture

Viral genomes, like all genomes, evolve and adapt to changes in environmental pressures. Furthermore, the rate of adaptation is specific to individual genes exposed to a particular selective environment (Bulach and Tribe, 1994). When conditions are radically altered, such as when baculoviruses are switched from *in vivo* to *in vitro* propagation, rapid changes in the phenotype may result as the selection of optimal genotypes takes place. An *in vitro* environment is radically different in many ways. This includes the use of a specific cell type for virus propagation, the form and supply of nutrients, and the use of BV as the sole means of genetic propagation.

Scaling up a baculovirus–insect cell process involves a large increase in volumetric scale and requires multiple passages of the virus for completion (Rhodes, 1996). A major difficulty of *in vitro* production of baculoviruses is the development of virus instability or the so-called passage effect. The passage effect can result from a variety of mutations, where a decrease of polyhedra production and virulence are the main characteristics. These mutations can have a significant effect on commercial production *in vitro*. The most common mutations are: defective interfering particles (DIPs) and few polyhedra (FP) mutants.

FP mutants are characterized by reduced polyhedra and enhanced production of BV, resulting in a selection of the FP phenotype during serial passage in cell culture (Hink and Vail, 1973; Mackinnon *et al*., 1974; Potter *et al*., 1976; Sohi *et al*., 1984; Chakraborty and Reid, 1999; Pedrini *et al*., 2004). The polyhedra that are produced have practically no occluded virions (*Figure 19.4*), resulting in low or no infectivity of the target pest.

Spontaneously generated FP mutants are well documented among wild-type baculoviruses that are serially passed in cell cultures, such as: *Autographa californica* NPV (AcMNPV) (Hink and Vail, 1973; Wood, 1980), *Galleria mellonella* NPV (GmMNPV) (Fraser and Hink, 1982), *Trichoplusia ni* NPV (TnMNPV) (Potter *et al*., 1976), *Lymantria dispar* NPV (LdMNPV) (Slavicek *et al*., 1995), *Orgyia pseudotsugata* NPV (OpMNPV) (Russell and Rohrmann, 1993), *Helicoverpa armigera* NPV (HaSNPV) (Chakraborty and Reid, 1999; Lua *et al*., 2002), *Bombyx mori* NPV (BmMNPV) (Katsuma *et al*., 1999), and *Spodoptera frugiperda* NPV (SfMNPV) (Pedrini *et al*., 2004).

The mutations have been observed predominantly within a specific DNA region (map units 36–37) on the AcMNPV (Fraser *et al*., 1983; Beames and Summers, 1988, 1989) and GmMNPV genomes (Fraser *et al*.,

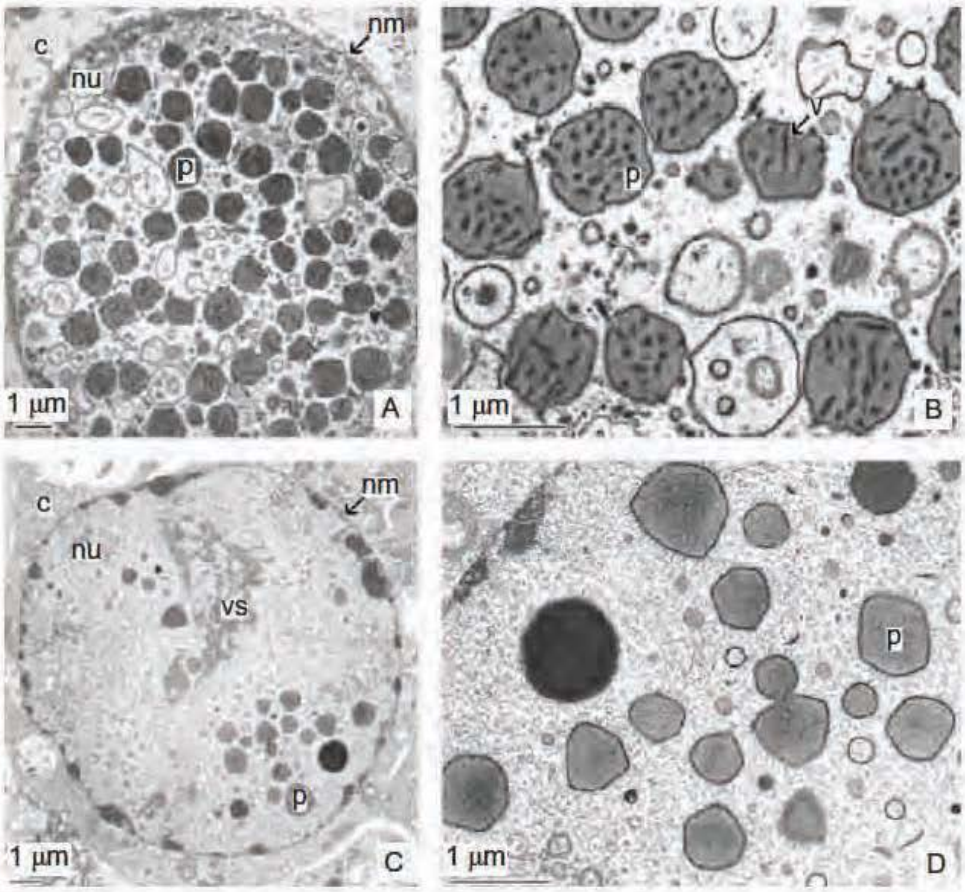


Figure 19.4

Electron micrographs illustrating the phenotypic characteristics of HaSNPV infection of *H. zea* cells grown in suspension cultures, comparing the use of the wild type MP isolate (A and B) with the plaque purified FP8AS (C and D). Micrographs B and D are higher magnifications of micrographs A and C, respectively, to illustrate in greater detail the ultrastructure of polyhedra and their embedded virions within the nucleus. The typical MP phenotype is that of many polyhedra within the nucleus and the presence of numerous virions embedded within these polyhedra, as exemplified by the wild type MP isolate. In contrast, the typical FP phenotype is that of considerably fewer polyhedra within the nucleus, with little or no virion embedment within these polyhedra, as shown by the FP8AS variant. c, nucleus; nm, nuclear membrane; nu, nucleus; p, polyhedra; v, virion; vs, virogenic stroma. From Pedrini, M.R.S., *et al.* Properties of a Unique Mutant of *Helicoverpa armigera* Single nucleocapsid Nucleopolyhedrovirus that Exhibits a Partial Many Polyhedra and Few Polyhedra Phenotype on Extended Serial Passaging in Suspension Cell Cultures. *In Vitro Cellular and Developmental Biology Animal*, Vol 41, pp 289 297. Copyright © 2005 by Society for In Vitro Biology. Reproduced with permission of the copyright owner.

1983, 1985). This region has the *25K FP* gene, which encodes a 25 kDa protein (Harrison and Summers, 1995; Braunagel *et al.*, 1999). This protein is essential for polyhedron formation and virion occlusion since deletion of this gene is sufficient to eliminate these processes (Beames and Summers, 1988, 1989, Wang *et al.*, 1989; Harrison and Summers, 1995, Braunagel *et al.*, 1999). These mutations have been correlated to large insertions of host DNA or deletions of the viral genome (Fraser and Hink,

1982; Fraser *et al*., 1983; Beames and Summers, 1988, 1989; Wang *et al*., 1989). However, for the HaSNPV (Lua *et al*., 2002) and LdMNPV (Slavicek *et al*., 1995; Bischoff and Slavicek, 1997b), point mutations or simple base pair insertions were observed within the *25K FP* gene. The FP mutation of HaSNPV and LdMNPV is likely to arise through a different mechanism from AcMNPV and GmMNPV (Fraser *et al*., 1983; Beames and Summers, 1988), but probably similar to both viruses (Lua *et al*., 2002).

When the virus has gone through a long period of multiple infection cycles, the ability to infect insect cells decreases due to the formation of defective interfering particles (DIPs). DIPs are found in most animal virus systems (Huang and Baltimore, 1977) and are well documented in many baculovirus systems that undergo *in vitro* serial passaging, such as: AcMNPV (Carstens, 1982; Kool *et al*., 1991; Lee and Krell, 1992; Pijlman *et al*., 2001), BmMNPV (Hashimoto *et al*., 1993; Yanase *et al*., 1998), SeMNPV (Heldens *et al*., 1996), *H. zea* baculovirus subgroup C (Burand and Wood, 1986; Chao *et al*., 1990), and HaSNPV (Pedrini *et al*., 2005). Some of these data are summarized in *Table 19.1*.

Pijlman *et al*. (2001) demonstrated that DIP generation and accumulation are intrinsic characteristics of baculovirus propagation in insect cell cultures, leading to a dramatic loss of BV titer. However, the origin of DIPs is not fully understood. Pijlman *et al*. (2001) proposed a mechanism involving continuous hetero- and homologous recombination of baculovirus DNA within insect cells, the deletion mutants of which are then selected for, depending on their relative replicative advantages. A high frequency of recombination between homologous baculoviruses in insect cell cultures has been demonstrated previously (Hajós *et al*., 2000). It was found that AcMNPV mutant virions lack around 44% of the viral genome, including polyhedra and DNA polymerase genes (Kool *et al*., 1991; Wickham *et al*., 1991). These particles containing only part of their

Table 19.1. Defective interfering particles (DIPs) for many baculovirus submitted to successive passages in insect cell cultures

Virus	Cell line	Native virus size (kb)	DIP size (kb)	% of native virus	Reference
A. californica NPV (AcMNPV)	*S. frugiperda* (Sf)	128	75	59	Carstens, 1982
A. californica NPV (AcMNPV)	*S. frugiperda* (Sf AE 21)	130	100; 85; 70	77; 65; 54	Lee and Krell, 1992
A. californica NPV (AcMNPV Bacmid bGFP)	*S. frugiperda* (Sf AE 21)	Not given	Not given	57	Piljman *et al.*, 2001
B. mori NPV (BmMNPV D1)	*B. mori* (BmN4)	Not given	Not given	74	Hashimoto *et al.*, 1993
S. exigua NPV (SeMNPV)	*S. exigua* (Se UCR1)	134	109	81	Heldens *et al.*, 1996
H. zea subgroup C Baculovirus (Hz 1)	*T. ni* (Tn 368)	228	176 204	77 89	Chao *et al*., 1990
H. armigera NPV (HaSNPV)	*H. zea* (Hz AM1)	133.4	115.4; 92.3	87, 7	Pedrini *et al*. 2005

genome need an intact virus as a helper (Lee and Krell, 1992), but replicate faster than standard virus, and consequently, increase their yield with continuous serial passage.

DIP formation in virus stocks can be avoided by infecting insect cells at a low multiplicity of infection (MOI). According to Wickham *et al*. (1991), a low MOI is used to minimize the probability of a DIP entering in a cell along with an intact helper virion. As a result, some cells receive only intact virus, others only defective virus, or no virus at all. Non-infected cells or cells infected only with defective virus will not produce any more virus, while cells infected with intact virus will produce more intact and infectious viruses than the co-infected cells. Therefore, the DIP fraction will decrease compared with the original inoculum. This causes an increase of virus titer.

Other genomic mutations can also affect the viral infectivity in caterpillars, such as: enhancing genes (E1 and E2) (Bischoff and Slavicek, 1997a; Popham *et al*., 2001), SeMNPV ORF35 (Se35) gene (Pijlman *et al*., 2001), and the SeMNPV ORF36 (Se36), designated as *per os infectivity factor* (pif) (Kikhno *et al*., 2002). An understanding of how these genes affect viral infectivity may help to develop culture strategies that avoid these mutations. This could improve the commercial prospects of biopesticides produced *in vitro*.

References

Adams JR, Bonami JR (1991), Atlas of Invertebrate Viruses, CRC Press, Boca Raton, FL, p. 684.

Agência Brasil (2004), Brasil tem o maior e melhor programa de controle biológico do mundo, Disponível em: Revista Biotecnologia Ciência & Desenvolvimento. Avail able at: http://www.biotecnologia.com.br/bionoticias/noticia.asp?id=224 (acessed September 2006).

Almeida AF, Silva SF, Macedo GR, Pedrini MRS (2005), Produção de biopesticida viral *Spodoptera frugiperda* MNPV utilizando células Sf9, XV Simpósio Nacional de Bioprocessos, Recife, PE.

Beames B, Summers MD (1988), Comparisons of host cell DNA insertions and altered transcription at the site of insertions in few polyhedra bacilovirus mutants, Virology 162:206 220.

Beames B, Summers MD (1989), Location and nucleotide sequence of the 25K protein missing from baculovirus few polyhedra (FP) mutants, Virology 168:344 353.

Belloncik S, Akoury WE, Cheroutre M (1997), Importance of cholesterol for nuclear polyhedrosis virus (NPV) replication in cell cultures adapted to serum free medium, In: Maramorosh K, Mitsubashi J (Eds), Invertebrate Cell Culture: Novel Directions and Biotechnology Applications, Science Publishers, Enfield, NH, pp. 141 147.

Bilimoria SL, Demirbag Z, Ng H (1992), Abortive cell culture infections of nuclear polyhedrosis viruses as model systems for host specificity, Pesquisa Agropecuária Brasileira 27:123 141.

Bischoff DS, Slavicek JM (1997a), Molecular analysis of an enhancing gene in the Lymantria dispar nuclear polyhedrosis virus, J. Virol. 71:8133 8140.

Bischoff DS, Slavicek JM (1997b), Phenotypic and genetic analysis of *Lymantria dispar* nucleopolyhedrovirus few polyhedra mutants: mutations in the 25K FP gene may be caused by DNA replication errors, J. Virol. 71:1097 1106.

Bonning BC, Hoover K, Duffey S, Hammock BD (1995), Production of polyhedra of

the Autographa californica nuclear polyhedrosis virus using the Sf21 and Tn5B1 4 cell lines and comparison with host derived polyhedra by bioassay, J. Invertebr. Pathol. 66:224 230.
Braunagel SC, Burks JK, Rosas Acosta G, Harrison RL, Ma H, Summers MD (1999), Mutations within the *Autographa californica* nucleopolyhedrovirus FP25K gene decrease the accumulation of ODV E66 and alter its intranuclear transport, J. Virol. 73:8559 8570.
Bulach D, Tribe D (1994), Tools for tracking insect virus variation in nature and during biopesticide manufacturing. Proceedings of the 1st Brisbane Symposium Bio pesticides: Opportunities for Australian Industry, 9 10, Brisbane, Australia.
Burand JP, Wood HA (1986), Intracellular protein synthesis during standard and defective Hz 1virus replication, J. Gen. Virol. 67:167 173.
Caron AW, Archambault J, Massie B (1990), High level recombinant protein produc tion in bioreactors using the baculovirus insect cell expression system, Biotechnol. Bioeng. 36:1133 1140.
Carpenter WM, Bilimoria SL (1983), A semi permissive nuclear polyhedrosis virus infection: characterization of infection kinetics and morphogenesis, Virology 130:227 231.
Carstens EB (1982), Mapping the mutation site of an *Autographa californica* nuclear polyhedrosis virus polyhedron morphology mutant, J. Virol. 43:809 818.
Carvalho RS, Nascimento AS, Matrangolo WJR (1999), Inseto exótico controla mosca das frutas, A Lavoura 628:40 43.
Castro MEB, Souza ML, Araujo S, Bilimoria, SL (1997), Replication of *Anticarsia gemmatalis* nuclear polyhedrosis virus in four lepidopteran cell lines, J. Invertebr. Pathol. 69:40 45.
Chakraborty S, Reid S (1999), Serial passage of a *Helicoverpa armigera* nucleopolyhe drovirus in *Helicoverpa zea* cell cultures, J. Invertebr. Pathol. 73:303 308.
Chakraborty S, Monsour C, Teakle R, Reid S (1999), Yield, biological activity, and field performance of a wild type *Helicoverpa* nucleopolyhedrovirus produced in H. zea cell cultures, J. Invertebr. Pathol. 73:199 205.
Chao YC, Hamblin M, Wood HA (1990), Physical map of Hz 1 baculovirus genome from standard and defective interfering particles, J. Gen. Virol. 71:1265 1270.
Elam P, Vail PV, Schreiber F (1990), Infectivity of Autographa californica nuclear polyhedrosis virus extracted with digestive fluids of *Heliothis zea*, *Estigmene acrea*, and carbonate solutions, J. Invertebr. Pathol. 55:278 283.
EMBRAPA (2002), Relatório Ambiental, EMBRAPA, Brasília, DF, Brazil, p. 67.
Faulkner P, Henderson JF (1972), Serial passage of a nuclear polyhedrosis disease virus of the cabbage looper (Trichoplusia ni) in a continuous tissue culture cell line, Virology 50:920 924.
Federici, B. A. (1986), Ultrastructure of baculoviruses, In: Granados RR, Federici BA (Eds), The Biology of Baculoviruses: Biological Properties and Molecular Biology, CRC Press, Boca Raton, FL, pp. 61 88.
Fraser MJ, Hink WF (1982), The isolation and characterization of the MP and FP plaque variants of *Galleria mellonella* polyhedrosis virus, Virology 117:366 378.
Fraser MJ, Smith GE, Summers MD (1983), Acquisition of host cell DNA sequences by baculovirus: relationship between host DNA insertion and FP mutants of Autographa californica and Galleria mellonella nuclear polyhedrosis viruses, Virology 47:287 300.
Fraser MJ, Brusca JS, Smith GE, Summers MD (1985), Transposon mediated mutagen esis of a baculovirus, Virology 145:356 361.
Friesen PD, Miller LK (2001), Insect viruses, In: Knipe DM, Howley PM (Eds), Fields' Virology, 4th Ed., Lippincott Williams & Wilkins, Philadelphia, pp. 599 621.
Godwin GP, Danner DJ, Gorfien SF (1996), Development of concentrated nutrient supplements to enhance recombinant protein expression in BEVS, BIOT 99,

Abstracts of the 211th American Chemical Society National Meeting, March 24 28, New Orleans, LA.
Goodman CL, McIntosh AH, El Sayed GN, Grasela JJ, Stiles B (2001), Production of selected baculoviruses in newly established lepidopteran cell lines, In Vitro Cell. Dev. Biol. (Animal) 37:374 379.
Granados RR, Federici BA (1986), The Biology of Baculoviruses, vol. 2, CRC Press, Boca Raton, FL.
Hajós JP, Pijnenburg J, Usmany M, Zuidema D, Závodszky P, Vlak JM (2000), High frequency recombination between homologous baculoviruses in cell culture, Arch Virol. 145:159 164.
Harrap KA (1972), The structure of nuclear polyhedrosis viruses. III Virus assembly, Virology 50:133 139.
Harrison RL, Summers MD (1995), Biosynthesis and localization of the Autographa californica nuclear polyhedrosis virus 25K gene product, Virology 208:279 288.
Hashimoto Y, Hatanaka E, Ishizaki A, Matsumoto T (1993), Production of defective genomes of the *Bombyx mori* nuclear polyhedrosis virus by serial passage through cultured cells, Jpn J. Appl. Entomol. Zool. 37:240 243.
Heldens JG, van Strien EA, Feldmann AM, Kulcsar P, Munoz D, Leisy DJ, Zuidema D, Goldbach RW, Vlak JM (1996), *Spodoptera exigua* multicapsid nucleopoly hedrovirus deletion mutants generated in cell culture lack virulence in vivo, J. Gen. Virol. 77:3127 3134.
Hink WF, Vail PV (1973), A plaque assay for titration of alfalfa lopper nuclear polyhedrosis virus in a cabbage lopper (TN 368) cell line, J. Inverteb. Pathol. 22:168 174.
Huang AS, Baltimore D (1977), Defective interfering animal viruses, In: Fraenkel Conrat H, Wagner RR (Eds), Comprehensive Virology: Regulation and Genetics, Plenum Press, New York, pp. 73 116.
IBGE (2005), Levantamento Sistemático da Produção Agrícola Disponível em. Avail able at: http://www1.ibge.gov.br/home/presidencia/noticias/noticia visualiza.php ?id noticia=423&id pagina=1 (accessed September 2006).
Ignoffo CM, Hostetter DL, Shapiro M (1974), Efficacy of insect viruses propagated in vivo and in vitro, J. Invertebr. Pathol. 24:184 187.
Jarvis AW (1997), Baculovirus expression vectors. In: Miller LK (Ed.), The Baculo viruses, Plenum Press, New York, pp. 389 431.
Katsuma S, Noguchi Y, Zhou CL, Kobayashi M, Maeda S (1999), Characterization of the 25K FP gene of the baculovirus *Bombyx mori* nucleopolyhedrovirus: implica tions for post mortem host degradation, J. Gen. Virol. 80:783 791.
Kikhno I, Gutierrez S, Croizier L, Croizier G, Ferber, ML (2002), Characterization of pif, a gene required for the per os infectivity of Spodoptera littoralis nucleopoly hedrovirus, J. Gen. Virol. 83:3013 3022.
King LA, Possee RD (1992), The Baculovirus Expression System: A Laboratory Guide, Chapman & Hall, London.
Kool M, Voncken JW, van Lier FL, Tramper J, Vlak JM (1991), Detection and analysis of *Autographa californica* nuclear polyhedrosis virus mutants with defective interfering properties, Virology 183:739 746.
Lee HY, Krell PJ (1992), Generation and analysis of defective genomes of *Autographa californica* nuclear polyhedrosis virus, J. Virol. 66:4339 4347.
Liu HS, Bilimoria SL (1990), Infected cell specific protein and viral DNA synthesis in productive and abortive infections of *Spodoptera frugiperda* nuclear polyhedrosis virus, Arch Virol. 115:101 113.
Lua LHL, Pedrini MRS, Reid S, Robertson A, Tribe DE (2002), Phenotypic and genotypic analysis of Helicoverpa armigera nucleopolyhedrovirus serially pas saged in cell culture, J. Gen. Virol. 83:945 955.

Lua LHL, Nielsen LK, Reid S (2003), Sensitivity of Helicoverpa armigera nucleopoly hedrovirus polyhedra to sodium dodecyl sulfate, Biol. Control 26:57 67.
Lynn DE (1994), Enhanced infectivity of occluded virions of Gypsy Moth nuclear polyhedrosis virus for cell cultures, J. Invertebr. Pathol. 63:268 274.
Lynn DE (1999), Comparison of cell line maintenance procedures on insect cells used for producing baculoviruses, In Vitro Cell Dev. Biol. (Animal) 35:248 251.
Lynn DE (2003a), Comparative susceptibilities of insect cell lines to infection by the occlusion body derived phenotype of baculoviruses, J. Invertebr. Pathol. 83: 215 222.
Lynn DE (2003b), Comparative susceptibilities of twelve insect cell lines to infection by three baculoviruses, J. Invertebr. Pathol. 82:129 131.
McIntosh AH, Ignoffo CM, Carlo M (1988), Effect of larval extract and protease K on the in vitro infectivity of Heliothis zea nonoccluded and alkali liberated virions, Appl. Ent. Zool. 23:175 180.
Mackinnon EA, Henderson JF, Stoltz DB, Faulkner P (1974), Morphogenesis of nuclear polyhedrosis virus under conditions of prolonged passage in vitro, J. Ultrastruct. Res. 49:419 435.
Maiorella B, Inlow D, Shauger A, Harano D (1988), Large scale insect cell culture for recombinant protein production, Bio Technology 6:1406 1410.
Miltenburger HG, Naser WL, Harvey JP, Huber J, Huger AM (1984), The cellular substrate: a very important requirement for baculovirus in vitro replication, Z. Naturforsch. 39:993 1002.
Moscardi F (1993), Soybean integrated pest management in Brazil, FAO Plant Protec tion Bulletin 41:91 100.
Moscardi F (1999), Assessment of the application of baculoviruses for control of *Lepidoptera*, Annu. Rev. Entomol. 44:257 289.
Moscardi F, Santos B (2005), Produção comercial do nucleopoliedrovirus de *Anticarsia gemmatalis Huber (Lep.: Noctuidae)* em laboratório, 9º Simpósio de Controle Biológico (Siconbiol 2005), Recife, PE.
Moscardi F, Souza ML (2002), Baculovírus para o controle de pragas: Panacéia ou realidade? Biotecnologia Ciência & Desenvolvimento 24:22 29.
Nguyen B, Jarnagin K, Williams S, Chan H, Barnett J (1993), Fed batch culture of insect cells: a method to increase the yield of recombinant human nerve growth factor (rhNGF) in the baculovirus expression system, J. Biotechnol. 31:205 217.
Pedrini MRS, Wolff JLC, Reid S (2004), Fast accumulation of few polyhedra mutants during passage of a *Spodoptera frugiperda* multicapsid nucleopolyhedrovirus (Baculoviridae) in Sf9 cell cultures, Ann. Appl. Biol. 145:107 112.
Pedrini MRS, Nielsen LK, Reid S, Chan LCL (2005), Properties of a unique mutant of *Helicoverpa armigera* single nucleocapsid nucleopolyhedrovirus that exhibits a partial many polyhedra and few polyhedra phenotype on extended serial passa ging in suspension cell cultures, In Vitro Cell Dev. Biol. (Animal) 41:289 297.
Pijlman GP, Born, EVD, Martens DE, Vlak JM (2001), Autographa californica baculoviruses with large genomic deletions are rapidly generated in infected insect cells, Virology 283:132 138.
Popham HJR, Bischoff DS, Slavicek JM (2001), Both *Lymantria dispar* nucleopoly hedrovirus Enhancin genes contribute to viral potency, J. Virol. 75:8639 8648.
Potter KN, Faulkner P, Mackinnon EA (1976), Strain selection during serial passage of *Trichoplusia ni* nuclear polyhedrosis virus, J. Virol. 18:1040 1050.
Rhodes DJ (1996), Economics of baculovirus insect cell production systems, Cyto technology 20:291 297.
Rohrmann GF (1999), Nucleopolyhedrovirus, In: Granoff A, Webster RG (Eds), Encyclopedia of Virology, Academic Press, New York, pp. 146 152.
Rollinson WD, Lweis FB, Waters WE (1965), The successful use of a nuclear polyhedrosis virus against the gypsy moth. J. Invertebr. Pathol. 7:515 517.

Russell RQL, Rohrmann GF (1993), A 25 kDa protein is associated with the envelopes of occluded baculovirus virions, Virology 195:532 540.
Santos B (2003), Avanços na produção massal de lagartas de *Anticarsia gemmatalis Hubner* 1818 (*Lepidoptera: Noctuidae*) infectadas com o seu vírus de poliedrose nuclear, em laboratório e do bioinseticida à base desse vírus, Tese de Doutorado, Universidade Federal do Paraná, Curitiba, PR.
Schlaeger EJ (1996), Medium design for insect cell culture, Cytotechnology 20:57 70.
Slavicek JM, Hayes Plazolles N, Kelly ME (1995), Rapid formation of few polyhedra mutants of *Lymantria dispar* multinucleocapsid nuclear polyhedrosis virus during serial passage in cell culture, Biol. Control. 5:251 261.
Slavicek JM, Mercer MJ, Kelly ME, Hayes Plazolles N (1996), Isolation of a Baculo virus variant that exhibits enhanced polyhedra production stability during serial passage in cell culture, J. Invertebr. Pathol. 67:153 160.
Sohi SS, Percy J, Arif BM, Cunningham JC (1984), Replication and serial passage of a singly enveloped baculovirus of *Orgyia leucostigma* in homologous cell lines, Intervirology 21:50 60.
Szewczyk B, Hoyos Carvajal L, Paluszek M, Skrzecz I, Souza LM (2006), Baculo viruses re emerging biopesticides, Biotechnol. Adv. 24:143 160.
Vail PV, Romine CL, Vaughn JL (1979), Infectivity of nuclear polyhedrosis virus extract with digestive juices, J. Invertebr. Pathol. 33:328 330.
Vicente JR, Gonçalves JS, Martin NB, Souza SAM (2003), Balança comercial dos agronegócios, Janeiro a Setembro de 2003. Available at: http://www.iea.sp.gov.br/out/verTexto.php?codTexto=1533 (acessed September 2006).
Volkman LE, Summers MD, Hsieh CH (1976), Occluded and non occluded nuclear polyhedrosis virus grown in Trichoplusia ni: comparative neutralization compara tive infectivity, and in vitro growth studies, J. Virol. 19:820 832.
Wang HH, Fraser MJ, Cary LC (1989), Transposon mutagenesis of baculoviruses: analysis of TFP3 lepidopteran transposon insertions at the FP locus of nuclear polyhedrosis viruses, Gene 81:97 108.
Wang P, Granados RR, Shuler ML (1992), Studies on serum free culture of insect cells for virus propagation and recombinant protein production, J. Invertebr. Pathol. 59:46 53.
Wickham TJ, Davis T, Granados RR, Hammer DA, Shuler ML, Wood HA (1991), Baculovirus defective interfering particles are responsible for variations in recom binant protein production as a function of multiplicity of infection, Biotechnol. Lett. 13:483 488.
Winstanley D, Crook NE (1993), Replication of Cydia pomonella granulosis virus in cell cultures, J. Gen. Virol. 74:1599 1609.
Winstanley D, O'Reilly DR (1999), Granuloviruses, In: Granoff A, Webster RG (Eds), Encyclopedia of Virology, 2nd Ed., Academic Press, New York, pp. 140 146.
Wood HA (1980), Isolation and replication of an occlusion body deficient mutant of the Autographa californica nuclear polyhedrosis virus, Virology 105:338 344.
Yanase T, Hashimoto Y, Matsumoto T (1998), Analysis of defective genomes of *Bombyx mori* nucleopolyhedrovirus generated by serial undiluted passage in cell culture, Acta Virol. 42:65 70.

Cell therapies and stem cells

20

Hamilton da Silva Jr and Radovan Borojevic

20.1 Introduction

Cell therapy is a medical procedure that rebuilds structures and restores functions of deteriorated tissues or organs by stem cells. This approach is feasible in treatments of tissue dysfunction generated by trauma, infectious disease, degenerative processes caused by natural fatigue or environmental insults, or a natural consequence of senescence. The cell-mediated regenerative medical interventions have a number of technical and biological limitations, which have stimulated new studies in biological processes and engineering. The advances in these two domains have created a multidisciplinary new field of biotechnology and bioengineering. The innovative work in this field has the potential for improving the quality of life of humans and significantly decreasing healthcare costs. The traditional use of cell therapy has been in bone marrow transplantation and treatment of autoimmune diseases. More recently, cell therapies have been applied with success in reparative surgery of soft tissues, skin and mucosa, peripheral nerve lesions, orthopedics including degenerative processes and trauma, angiogenesis, heart diseases, and stroke.

The application of cell therapies is based on histological (homotopic or heterotopic) and immunological (autologous transplants, allotransplants or xenotransplants) principles. Homotopic therapies are defined as procedures that use cells for repair and regeneration of the tissue from which they have been harvested. Good examples of homotopic therapies are: skin repair with *in vitro* expanded epidermal cells, isolation and implantation of pancreatic Langerhans islets (Eliaschewitz *et al.*, 2004), and bone marrow aspiration to restore the hematopoietic tissue (Shizuru *et al.*, 2005). Heterotopic therapies use stem cells obtained from a source different from that in which they are used. The most frequent source of stem cells used in repair of peripheral tissues is bone marrow. However, it is now known that many tissues, such as adipose or blood vessel walls, harbor pluripotent stem cells that can be used in repair and regeneration. This procedure depends upon the pluripotency of stem cells or their transdifferentiation, and the underlying molecular mechanisms are not well understood. The paracrine effect of implanted cells that may activate the resident cell progenitors

has to be taken into consideration, as well as modification of the intercellular environment following cell injection.

Autologous transplants involve the transfer of cells, tissues, or organs from the same individual. Conversely, allotransplants or allogeneic transplants use cells from different individuals within the same species. Xenotransplants involve transplantation or temporary use of organs or tissues from a different species (e.g. porcine to human). In cases of allotransplants and xenotransplants, the implanted cells are necessarily identified as foreign by the receptor's immune system, which triggers an immune rejection process. In the case of bone marrow transplantation, the new immune system will be formed by transplanted cells of the donor. Therefore, the rejection will be donor versus receptor (graft versus host disease, GVHD). Allotransplants and xenotransplants are maintained by immunosuppressive therapies, which are aggressive and have to be maintained for long periods. Notwithstanding this drawback, allogeneic transplants are necessary to correct pathologies associated with modifications in the genome, mutations leading to cancer, or in which the patient's cells are not able to promote repair and tissue regeneration.

Heterotopic autologous therapies are at present the most frequently explored in basic, preclinical, and clinical research. Since 1990, the multipotency of adult stem cells, mainly from the bone marrow, has allowed the possibility of repair and regeneration of different tissues using the cells of the patient. The best example of heterotopic autologous therapy is the use of stem cells from the bone marrow in the treatment of cardiac ischemia. The rationale for this application is the capacity of such stem cells to differentiate *in situ* into cell lineages of the receptor site. The main goal of this kind of therapy is to offer tissue repair as close as possible to the physiologically normal tissue without restrictions of histocompatibility, which is a problem in allotransplants and xenotransplants. It is hoped that in the future these therapies will be able to restore an entire organ, such as pancreas, heart, or kidney, without the immune rejection and the required immunosuppressive treatments.

20.2 Primary material

The concept of cellular therapy involves manipulation and use of cells as a primary material, and this requires extensive knowledge of their structure and function (Orkin, 2000). A cell from any part of an organism does not exist as an isolated entity. It coexists in a three-dimensional system, highly organized and physiologically dependent upon the reciprocity of cell–cell and extracellular matrix–cell interactions. The understanding of all the elements related to the organization of a tissue structure is the major challenge for biological engineering. The tissue structures must be reconstructed, or at least equivalent structures have to be generated, to grant tissue function, using essentially three elements: the cells, the molecular mediators that promote and control cell growth, differentiation and tissue integration, as well as the three-dimensional matrices or scaffolds that provide the spatial organization.

20.2.1 Stem and mature cells

Any stem cell is defined by being able to maintain its own population by self-renewal and to differentiate into one or several types of mature fully differentiated cells. Stem cell proliferation can be modulated between low and high proliferation kinetics. Embryonic stem cells have a high proliferation rate, since they must rapidly construct an entire new organism. Adult stem cells are generally quiescent or proliferate slowly, but they can be mobilized upon demand, and induced to actively proliferate, as occurs with hematopoietic stem cells (Kondo *et al.*, 2003). The properties of stem cells are associated with their ability to undergo symmetrical or asymmetrical division (*Figure 20.1*). Classical symmetrical divisions are operational in the self-renewal or expansion of the original stem cell pool. Asymmetrical division depends upon the existence of intracellular segregated microdomains and organization of genetic elements, as observed in ovocytes. This organization allows micro-compartmentalized elements to be partially or totally segregated during cytoplasm division upon mitosis. Thus, a stem cell (mother cell) in mitotic division can generate an identical unit and another one with a different profile (daughter cell), which is generally engaged and committed into differentiation along a required pathway.

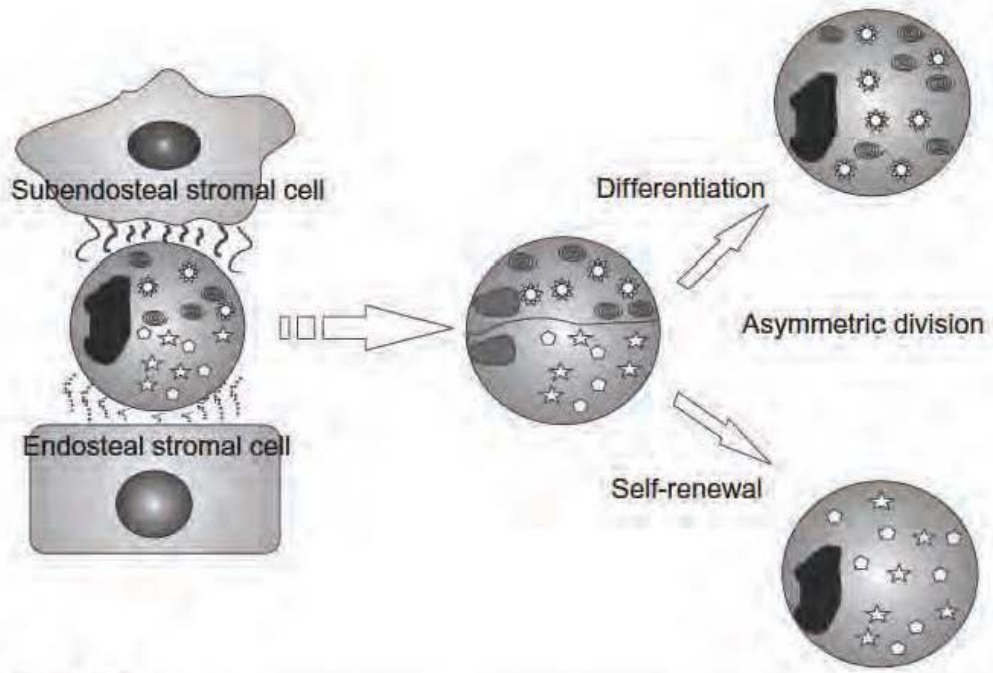


Figure 20.1

Schematic representation of an asymmetric cell division. This figure shows a hematopoietic stem cell under the influence of cell components within its niche. Between the stromal endosteal and subendosteal cell layers, the hematopoietic stem cell is stimulated into distinct polarities that split cytoplasmic components into microdomains. The cell division that is able to promote a different distribution of these intracellular elements, generates distinct offspring. The asymmetric division explains the phenomena of simultaneous self renewal and differentiation.

Stem cells can be characterized into three main classes: embryonic, germinal, and somatic or adult stem cells. Embryonic stem cells (ESCs) are derived from early stages of an embryo, from the morula, or from the inner cell mass of the blastocyst. Both are able to produce an entire organism. From the morula, the source of cells can be derived during blastic cleavages, which occur just after fertilization. Each cell up to the stage of 2 cells in humans, and up to 32 cells in animals, depending upon the species, is able to generate a complete new embryo. On the other hand, stem cells can be harbored by isolating the inner cell mass of blastocysts. This stage corresponds to the period of implantation into the uterus. In humans, they may be obtained from left-over embryos generated by *in vitro* fertilization.

The cells of the inner cell mass can take part in the generation of all the tissues with the exception of the extra-embryonic structures such as trophoblast and placenta. They are the main target of the worldwide research on ESCs. To obtain these cells requires disintegration of the blastocyst. This fact has generated ethical and religious questions concerning human embryos.

Moreover, scientists are still concerned about how to control the high capacity of ESCs for proliferation and their apparently erratic differentiation. The goal is to be able to control maturation towards a predetermined cell lineage. These issues have to be solved before the use of ESCs in humans, particularly because of their high tendency to develop into teratocarcinomas.

The germinal stem cells or EGCs (embryonic germ cells) are found in reproductive tissues during embryo development. They populate gonads that harbor progenitors of gametes. Similar cells are found in male adult gonads. Apparently, they have the potential to generate multiple cell lineages, but the disadvantage for therapy is the difficulty of obtaining these cells, which would involve invasive surgery during embryo development. This is associated with further ethical questions.

Cells derived from an embryo implanted into a recipient are recognized as non-self, and upon transplantation they are identified by the recipient's immune system. Thus, transplantation causes rejection, and this limits their use in cell therapy. Advances in reproductive techniques have emerged recently with the possibility of therapeutic cloning as a new option for cell therapy. The proposal is to substitute the nucleus of an ovocyte or an embryonic cell established *in vitro*, with the nucleus of a somatic cell from the recipient, generating new ESC lineages. The ovocyte or embryonic progenitor cells will be able to combine the capacity to generate any tissue within the host genome, with the appropriate immunologic characteristics to prevent rejection. However, the defects of the genome will also be transferred, thus preventing the use of this technique for genomic defects. Nevertheless, the extensively manipulated nuclei generally develop unpredictable random chromosomal defects, which may modify the cellular phenotypes. However, in view of the size of the genome and the interactivity of its diverse regions, an assessment of the potential dangers of any alteration is not feasible, and the medical risk of using progenitor cells generated by cloning in human

beings is unacceptably high. Furthermore, human cloning is technically difficult and also involves many religious and ethical dilemmas. Therefore, somatic or adult stem cells seem to be the best current solution for cellular therapies.

Somatic (from the Greek *somatikos* = bodily) stem cells are present in almost all the tissues (Jiang *et al.*, 2002a). The most well-known system is the hematopoietic tissue. The hematopoietic system in adults is lodged within the bone marrow and its stem cells are specifically located in the endosteal region, that is, close to the internal bone surface. Moreover, bone marrow has at least three stem cell lineages: hematopoietic, mesenchymal, and endothelial stem cells (*Figure 20.2*). Among these, mesenchymal stem cells display the broadest capacity for differentiation. Mesenchymal cells are associated with the inner cell mass and are not in direct contact with the external environment. Although these cells are most frequently of mesodermal origin, they can also be derived from the ectoderm and endoderm, in a process termed the ''epithelio-mesenchymal transition.'' Mesenchymal stem cells are able to restore tissue structural elements, called the cellular stroma and the corresponding extracellular matrix. They influence differentiation of other stem cells, as described below. Descriptions of many mesenchymal cell populations in different tissues can be found in the scientific literature, but there are controversies as to whether they belong to different lineages (*Figure 20.2*). The fact that mesenchymal cell progenitors can circulate in blood makes this question even more difficult. Mesenchymal stem cells are apparently pluripotent and, therefore, they can generate many cell types, with the possible exception of germline cells (Jiang *et al.*, 2002b).

Studies of stem cell progression towards the completely differentiated mature cells have already identified several intermediary precursors, organized in a cascade (Shizuru *et al.*, 2005). The best known and studied cellular differentiation cascade is the hematopoietic system (*Figure 20.3*). Within hematopoiesis, it is possible to identify many intermediary precursors between the hematopoietic stem cell and mature blood cells. This identification is based mainly on the phenotypic profile of cellular surface proteins, using flow cytometry as the main tool. This is a relatively simple technique that involves coupling a monoclonal antibody (mAb) with a fluorescent marker (fluorochrome). In this way, diverse cellular markers can be combined and thus a cellular subpopulation can be defined, as shown in *Figure 20.3*.

The ideal protocol for tissue regeneration is the use of autologous cells in the heterotopic context. This approach allows the stem cells to produce a tissue or organ for autologous repair and tissue regeneration. The first step is to define the primary source and to identify the cells to be transplanted. Later, it is necessary to ensure that the transplanted cells recognize the target tissue and acquire the specific functions. This is the object of cell therapy *in vivo*, or bioengineering *ex vivo* or *in vitro*. It is necessary to understand the physiology of the cells to be generated. That is: how, where, when, and what it is necessary to do to convert the stem cells into functional differentiated tissue cells.

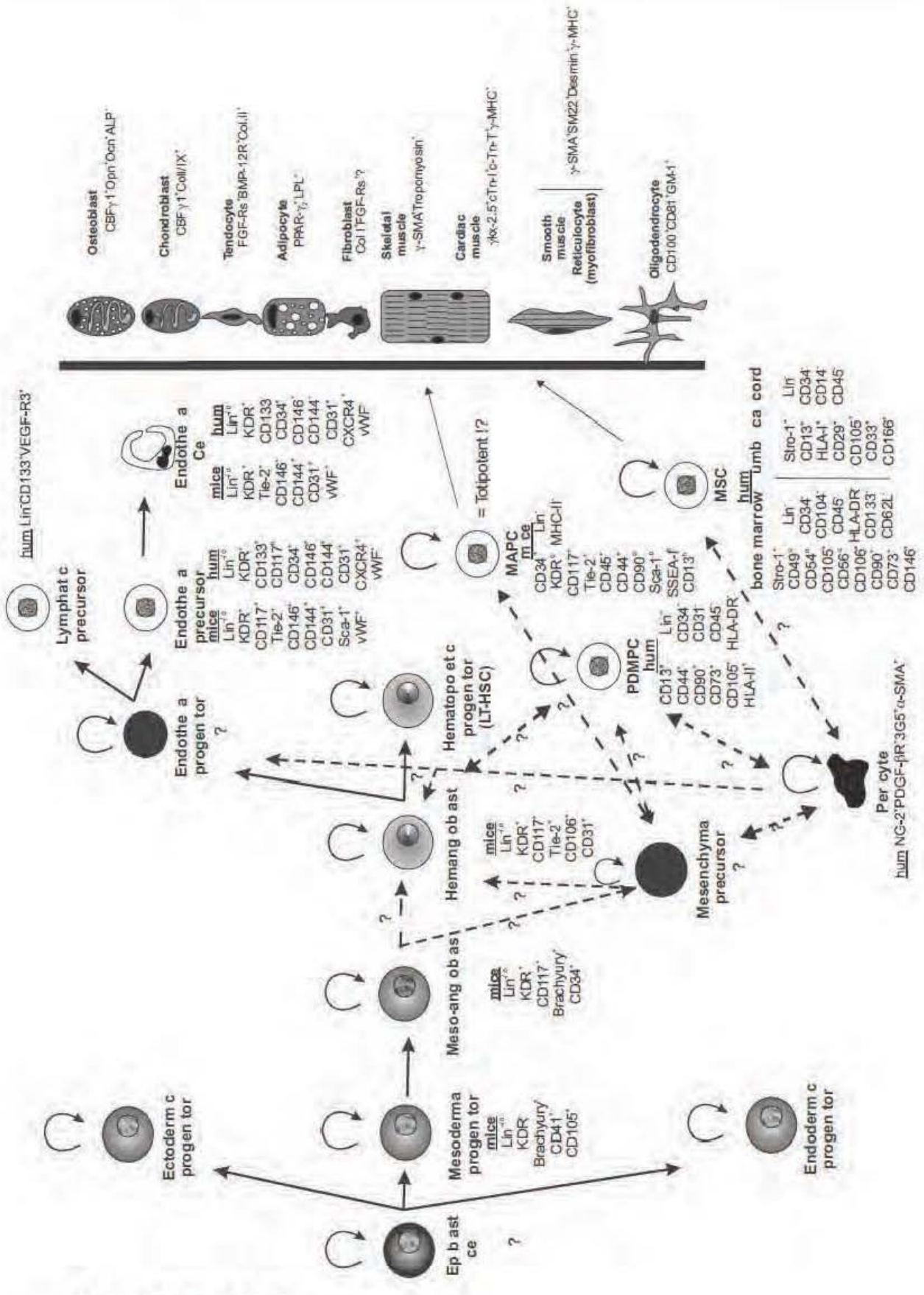

Osteoblast
CBF-γ1⁺Opn⁺Ocn⁺ALP⁺
Chondroblast
CBF-γ1⁺CollII/IX⁺
Tendocyte
FGF-Rs⁺BMP-12R⁺Col.I⁺
Adipocyte
PPAR-γ2⁺LPL⁺
Fibroblast
Col I⁺FGF-Rs⁺?
Skeletal muscle
γ-SMA⁺Tropomyosin⁺
Cardiac muscle
γMx-2.5⁺cTn-I⁺c-Tn-T⁺γ-MHC⁺
Smooth muscle
Reticulocyte (myofibroblast)
γ-SMA⁺SM22⁺Desmin⁺γ-MHC⁺
Oligodendrocyte
CD100⁺CD81⁺GM-1⁺
hum Lin⁻CD133⁺VEGF-R3⁺
Lymphatic precursor
Endothelial precursor
mice Lin⁻/⁰ KDR⁺ CD133⁺ CD117⁺ Tie-2⁺ CD146⁺ CD144⁺ CD31⁺ Sca-1⁺ vWF⁺ CXCR4⁺
hum Lin⁻/⁰ KDR⁺ CD133 CD117⁺⁺ CD34⁺ CD146⁺ CD144⁺ CD31⁺ vWF⁺ CXCR4⁺
Endothelial Ce
mice Lin⁻/⁰ KDR⁺ Tie-2⁺ CD146⁺ CD144⁺ CD31⁺ vWF⁺
hum Lin⁻/⁰ KDR⁺ CD133 CD34⁺ CD146⁺ CD144⁺ CD31⁺ CXCR4⁺ vWF⁺
Endothelial progenitor ?
Hematopoietic progenitor (LT-HSC)
= Totipotent !?
MAPC m ce
CD34⁺ Lin⁻ KDR⁺ MHC-I⁺ CD117⁺ Tie-2⁺ CD45⁻ CD44⁺ CD90⁺ Sca-1⁺ SSEA-f⁻ CD13⁺
PDMPC hum
CD13⁺ Lin⁻ CD44⁺ CD34⁻ CD90⁺ CD31⁻ CD73⁺ CD45⁻ CD105⁺ HLA-DR⁻ HLA-I⁺
MSC
hum bone marrow
Lin⁻ CD34⁻ CD104⁻ CD105⁺ CD45⁻ CD56⁺ HLA-DR⁻ CD106⁺ CD133⁻ CD90⁺ CD62L⁻
Stro-1⁺ CD49⁺ CD54⁺⁺ CD105⁺ CD56⁺ CD106⁺ CD90⁺ CD73⁺ CD146⁺
hum umb ca cord
Lin⁻ CD34⁻ Stro-1⁺ CD13⁺ HLA-I⁺ CD29⁺ CD105⁺ CD33⁺ CD166⁺
Lin⁻ CD34⁻ CD14⁻ CD45⁻
Hemangioblast
mice Lin⁻/⁰ KDR⁺ CD117⁺ Tie-2⁺ CD106⁺ CD31⁺
Mesenchymal precursor ?
Pericyte
hum NG-2⁺PDGF-βR⁺3G5⁺α-SMA⁺
Mesoangioblast
mice Lin⁻/⁰ KDR⁺ CD117⁺ Brachyury⁺ CD34⁺
Mesodermal progenitor
mice Lin⁻/⁰ KDR⁺ Brachyury⁺ CD41⁺ CD105⁺
Ectodermal progenitor
Endodermal progenitor
Epiblast cells
?

Figure 20.2

Schematic representation of the mesenchyma and endothe ia cascade of differentiation in a murine mode (mice) and in humans (hum). A mesenchyma and endothe ia ce s are negative for the antigen CD45. Based on this, the dynamics of surface antigen expression a ong deve opment of different mature ce s derived from the mesenchyma and endothe ia systems can be observed. Rounded arrows indicate se f-renewa potentia . Smooth, thinner arrows indicate directions of ce u ar differentiation, and dotted arrows indicate possib e hierarchies, but yet to be proved experimenta y. The question marks indicate ack of data on the pathway or on ce u ar identity. The identification of CDs (c usters of differentiation) and other antigen ce markers can be found in the ist of abbreviations: LT-HSC, ong-term hematopoietic stem ce ; EC, endothe ia ce ; PDMPC, p acenta -derived mesenchyma progenitor ce ; MSC, mesenchyma stem ce ; MAPC, mesenchyma adu t progenitor ce .

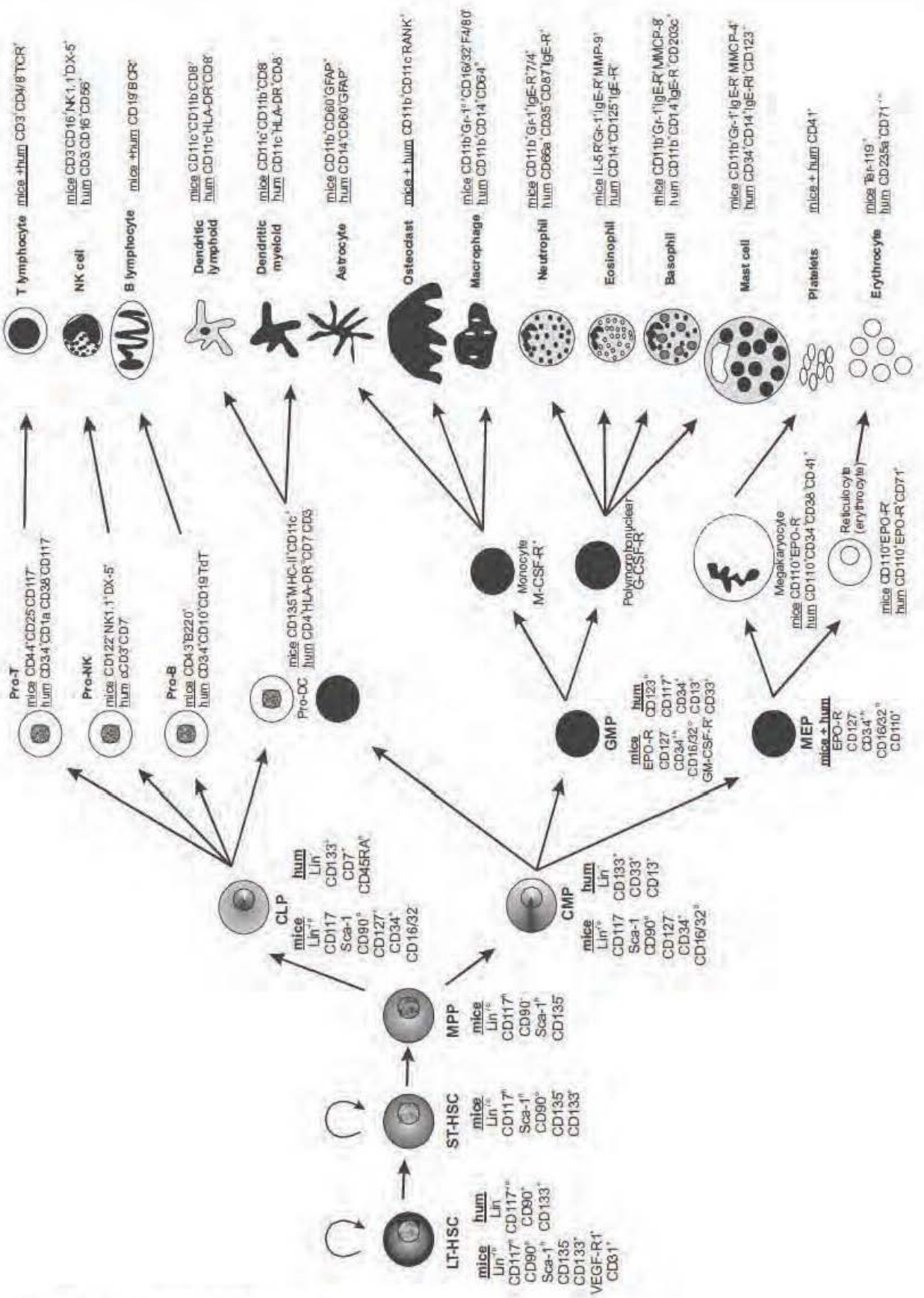

LT-HSC
mice Lin- CD117+ CD90+ Sca-1+ CD135- CD133- VEGF-R1+ CD31+
hum Lin- CD117+ CD90+ CD133+
ST-HSC
mice Lin- CD117+ Sca-1+ CD90+ CD135- CD133-
MPP
mice Lin- CD117+ CD90- Sca-1+ CD135+
CLP
mice Lin- CD117 Sca-1 CD90 CD127+ CD34+ CD16/32-
hum Lin- CD133+ CD7+ CD45RA+
CMP
mice Lin- CD117 Sca-1 CD90 CD127- CD34+ CD16/32-
hum Lin- CD133+ CD33+ CD13+
GMP
mice EPO-R- CD127- CD34+ CD16/32+ GM-CSF-R+ CD33+
hum CD123+ CD117+ CD34+ CD13+
MEP
mice + hum EPO-R- CD127- CD34+ CD16/32- CD110-
Pro-T
mice CD44+CD25+CD117+
hum CD34+CD1a+CD38-CD117+
Pro-NK
mice CD122+NK1.1+DX-5+
hum αCD3-CD7+
Pro-B
mice CD43+B220+
hum CD34+CD10+CD19+TdT+
Pro-DC
mice CD135+MHC-II+CD11c+
hum CD4+HLA-DR+CD7-CD3-
Monocyte M-CSF-R+
Polymorphonuclear G-CSF-R+
Megakaryocyte
mice CD110+EPO-R+
hum CD110+CD34-CD38+CD 41+
Reticulocyte (erythrocyte)
mice CD110+EPO-R+
hum CD110+EPO-R+CD71+
T lymphocyte mice +hum CD3+CD4+8+TCR+
NK cell mice CD3-CD16+NK1.1+DX-5+ hum CD3-CD16+CD56+
B lymphocyte mice +hum CD19+BCR+
Dendritic lymphoid mice CD11c+CD11b-CD8+ hum CD11c+HLA-DR+CD8+
Dendritic myeloid mice CD11c+CD11b+CD8- hum CD11c+HLA-DR+CD8-
Astrocyte mice CD11b+CD60+GFAP+ hum CD14-CD60+GFAP+
Osteoclast mice + hum CD11b+CD11c+RANK+
Macrophage mice CD11b+Gr-1+/-CD16/32+F4/80+ hum CD11b+CD14+CD64+
Neutrophil mice CD11b+Gr-1+IgE-R-7/4+ hum CD66a+CD35+CD87IgE-R-
Eosinophil mice IL-5Rα+Gr-1+IgE-R+MMP-9+ hum CD14-CD125+IgE-R+
Basophil mice CD11b+Gr-1+IgE-R+MMCP-8+ hum CD11b+CD14-IgE-R+CD203c+
Mast cell mice CD11b+Gr-1+IgE-R+ MMCP-4+ hum CD34+CD14-IgE-Rl+CD123+
Platelets mice + hum CD41+
Erythrocyte mice Ter-119+ hum CD235a+CD71+/-

Figure 20.3

Schematic representation of the hematopoietic cascade in mice and human (hum). A the ce s of the hematopoietic system are positive for antigen CD45. Based on this, the dynamics of surface antigen expression a ong deve opment of different mature ce s derived from the hematopoietic stem ce system can be observed. Rounded arrows indicate se f-renewa potentia . Smooth thinner arrows indicate directions of ce u ar differentiation. The identification of CDs (c usters definition) and other antigen ce markers are isted in the g ossary. (LT-HSC - ong term hematopoietic stem ce ; ST-HSC - short term hematopoietic stem ce ; MPP - mu tipotent progenitor; CLP - common ymphoid precursor; CMP - common mye oid precursor; GMP - granu ocyte-monocyte precursor; MEP - megacaryocyte-erythrocyte precursor; T - T ymphocyte; B - B ymphocyte; NK - natura ki er ce ; DC - dendritic ce).

20.2.2 Tissue environment and specific niches

The three-dimensional organization of a tissue is fundamental for the physiology and behavior of any cell. The local and peculiar structure of a tissue is called the tissue microenvironment (Spradling *et al.*, 2001). The spatial architecture around a cell is associated with the interstitial components, or extracellular matrix, which consist of structural glycoproteins, carbohydrates (collagens, elastin, proteoglycans, glycosaminoglycans), and adhesion compounds (fibronectin, laminin, thrombospondin, etc.), permeated by biological fluids. The cells have a broad range of accessory surface elements, consisting of the glycocalyx, rich in glycoproteins and glycolipids that interact with extracellular matrix. Finally, intercellular mediators such as hormones, cytokines, and inflammatory and growth factors allow cellular communication and proliferation control. These mediators interact with the intercellular matrix, which can generate gradients or hot spots with high concentrations of molecules that can interact with cell receptors and provide information on the quality of the tissue and on the necessity to promote repair or regeneration. Cell differentiation is a result of the combination of all these elements and interactions. The set of these molecular interactions characterizes the tissue microenvironment and this organization gives identity to a tissue. Since an ectopically transferred cell can modify its physiological activity in response to the new microenvironment, a stem cell of a heterotopic origin differentiates according to the environmental stimulus (Kai and Spradling, 2003).

Since tissues are spatially ordered by interfaces with solid support structures, blood vessels or external surfaces, the interactions among different types of cells determine the behavior of cells introduced during cell therapy. These interactions control differentiation and facilitate the formation of functional structures. It is important that a fraction of transplanted cells keeps the original properties of immature or stem cells, in order to be able to guarantee the physiological turnover, maintenance of tissue homeostasis, and natural renewal. In this context, the intercellular mediators, such as the chemokines, can guide the migration of the transplanted cell, directing it into an appropriate niche. The molecular composition of these niches allows some of the stem cells to maintain their pluripotency, while others respond to the demands for new differentiated cells. In the bone marrow, this niche is composed mainly of osteoblasts. The physical contact of these osteoblasts with hematopoietic stem cells is necessary to support hematopoiesis (Calvi *et al.*, 2003; Zhang *et al.*, 2003; Balduino *et al.*, 2005; Taichman, 2005).

Once out of the microenvironment, the absence of specific signals for the retention of multipotency and self-renewal inevitably induces irreversible and specific differentiation, leading to the generation of terminally differentiated cells. Stem cells thus loose their ''stemness'' and their primitive or undifferentiated identity (reviewed in Cabrita *et al.*, 2003). The understanding of fine controls for the maintenance of stemness is the primary goal for the development of systems that can sustain expansion of stem cells. Currently, the great challenge is to understand the complex system involved in the modulation of the intrinsic genetic programs of a stem cell. Once this information is available, it may be possible by

bioengineering to construct entire fragments of tissues or organs in the laboratory. These constructs would then be available for implantation into patients who need repair or regeneration of lost tissues following trauma or degeneration.

20.3 Applications

Many scientific groups are now working on cell therapies based on stem cells. It is difficult to imagine all the possible clinical applications that could be developed considering the great potential and plasticity of these cells. Medicine still lacks immediate and efficient solutions for situations of trauma, tissue dysfunction, or degeneration. Current knowledge is such that it is possible to produce the necessary cell mass *in vitro* for therapy, or to stimulate growth of tissue cells *in vivo*. It is also possible to construct in the laboratory, three-dimensional extracellular structures able to produce biological interactions among cellular elements.

20.3.1 Bioexpansion and biostorage

Finding a way around the shortage of primary material is one of the major challenges for bioengineering. The most widely studied system is the bone marrow. Hematopoietic stem cells comprise approximately 0.02% of the total cells obtained in a good bone marrow aspirate. A similar fraction is obtained in an umbilical cord blood sample, and a single sample can be used for transplantation in a 40 kg patient (Cohen and Nagler, 2004). Current methods proposed for *in vitro* hematopoietic stem cell expansion still represent a complex problem, since induction of proliferation is associated with the loss of stemness. By contrast, skin therapies are performed with a small healthy biopsy from the patient, since dissociated, isolated cells can be expanded *ex vivo*. These expanded cells are useful for regeneration or fulfilling other therapeutic procedures. The same principle can be applied to mesenchymal cells, which can be expanded *in vitro* to regenerate bone tissue.

Currently, several strategies for progenitor cell expansion already exist. However, the most advanced protocols for hematopoietic cell expansion still show limited success. Tests with small volumes may work with conventional culture systems for proliferating blood cells such as lymphocytes, in T-flasks under a carbon dioxide (CO_2)-rich atmosphere, while higher volumes are generally used in controlled bioreactors (Noll *et al.*, 2002; Cabrita *et al.*, 2003). In these systems, fine control is necessary for all the physicochemical factors, such as pH, temperature, trace elements, cytokines (Noll *et al.*, 2002). Each cell type requires specific conditions.

The key to expanding a stem cell population is to understand the requirements for proliferation without inducing differentiation. In the case of self-renewal some elements seem to be essential. Examples are the homeotic gene HOXB4, the cytokines FLT-3L (fms-related tyrosine kinase ligand), LIF (leukemia inhibitor factor), interleukin (IL)-3, VEGF (vascular endothelial growth factor), and proteins of the WNT and

NOTCH families, with their ligands (reviewed in Noll *et al.*, 2002; Cabrita *et al.*, 2003; Sorrentino, 2004).

Once it is possible to expand a clinically defined and safe cell lineage for transplant, it is also necessary to be able to store the viable cells for subsequent attempts or procedures. This can avoid further surgery on the same patient. This demands the development of crypreservation methods for cells and tissues, involving a tissue bank (blood, skin, bones, cornea, bone marrow, umbilical cord blood, etc.). Cryopreservation must be efficient for long periods of storage, since the frozen cells may be required years after the initial deposit.

20.3.2 Bioengineering

Bioengineering and biomimetics seek to develop tissue scaffolds similar to the natural ones, in terms of composition and biological behavior. Efforts are being made to develop products and processes that can substitute tissue that is biocompatible and allows biointegration.

These objectives are being sought for through distinct models of reparative cell therapies. For example, hydroxyapatite (HA), a natural bone constituent, can be synthesized to mold or to fill bone (Marcacci *et al.*, 1999a, 1999b). The rational strategy for the use of HA is osteoconduction and osteoinduction, since it is found in the normal bone tissue and elicits both adhesion and proliferation of bone cells. This fact also favors the final goal of cell therapy in the bone system, in which the progenitors are mesenchymal cell that can differentiate into osteoblasts responsible for the desired ossification. In the process of production of HA, it is possible to create a microporous texture that serves to facilitate the mesenchymal cell repopulation with autologous bone marrow source. Moreover, this composition represents an optimum biomimetic system in restorative therapies. Therefore, it can be produced on an industrial scale, and the biomaterial interacts well with autologous cell samples.

Another promising example is the use of composite dermo-epidermis plates whose dermis layer comes from plastic surgery waste or post-mortem donation. The skin fragment can be acellularized to eliminate the histo-incompatible cellular components from donor origin. The process gives an acellular skin structure that can be reconstituted with autologous epithelial stem cells or autologous keratinocytes *in vitro*. The final dermo-epidermal graft is appropriate to restore surfaces after severe burns or removal of tattooing, and to fill cavities in plastic and odontological surgeries.

A number of other processes are in continuous development. Clinical applications of these therapies in the urogenital and cardiovascular systems, in peripheral and central nervous systems, pancreas, joint cartilage restoration, etc., are being studied. However, almost all procedures suffer from a common limitation: the availability of donor cells. Cell therapies have to begin with a relatively high number of cells, and stem cells, irrespective of their origin, are always a minor subpopulation in tissues.

20.4 Conclusions and perspectives

Reparative medicine and bioprocess engineering are major issues for current applied biotechnology. Development in these areas is advancing towards the use of stem cells for therapy. However, the elements and mechanisms involved in the intrinsic and extrinsic biological control of these cells are poorly known. All clinical situations performed with autologous transplants of stem cells up to now have generally demonstrated improvement, or total or partial recovery of tissue function. Refining techniques in these therapeutic procedures will be necessary to better characterize and to understand the cellular subpopulations that participate in restoring the normal physiology of tissues. Concomitantly, engineering of biomaterials and the associated nanotechnology are appearing as important tools to speed up this scientific knowledge. The ultimate goal is to be able to totally or partially generate an organ with normal physiology, in a short time period.

References

Balduino A, Hurtado SP, Frazao P, Takiya CM, Alves LM, Nasciutti LE, El Cheikh MC, Borojevic R (2005), Bone marrow subendosteal microenvironment harbours functionally distinct haemosupportive stromal cell populations, Cell Tissue Res. 319:255 266.

Cabrita GJ, Ferreira BS, da Silva CL, Goncalves R, Almeida Porada G, Cabral JM (2003), Hematopoietic stem cells: from the bone to the bioreactor, Trends Biotechnol. 21:233 240.

Calvi LM, Adams GB, Weibrecht KW, Weber JM, Olson DP, Knight MC, Martin RP, Schipani E, Divieti P, Bringhurst FR, Milner LA, Kronenberg HM, Scadden DT (2003), Osteoblastic cells regulate the haematopoietic stem cell niche, Nature 425:841 846.

Cohen Y, Nagler A (2004), Umbilical cord blood transplantation how, when and for whom?, Blood Rev. 18:167 179.

Eliaschewitz FG, Aita CA, Genzini T, Noronha IL, Lojudice FH, Labriola L, Krogh K, Oliveira EM, Silva IC, Mendonca Z, Franco D, Miranda MP, Noda E, de Castro LA, Andreolli M, Goldberg AC, Sogayar MC (2004), First Brazilian pancreatic islet transplantation in a patient with type 1 *diabetes mellitus*, Trans plant. Proc. 36:1117 1118.

Jiang Y, Vaessen B, Lenvik T, Blackstad M, Reyes M, Verfaillie CM (2002a), Multi potent progenitor cells can be isolated from postnatal murine bone marrow, muscle, and brain, Exp. Hematol. 30:896 904.

Jiang Y, Jahagirdar BN, Reinhardt RL, Schwartz RE, Keene CD, Ortiz Gonzalez XR, Reyes M, Lenvik T, Lund T, Blackstad M, Du J, Aldrich S, Lisberg A, Low WC, Largaespada DA, Verfaillie CM (2002b), Pluripotency of mesenchymal stem cells derived from adult marrow, Nature 418:41 49.

Kai T, Spradling A (2003), An empty Drosophila stem cell niche reactivates the proliferation of ectopic cells, Proc. Natl Acad. Sci. U S A 100:4633 4638.

Kondo M, Wagers AJ, Manz MG, Prohaska SS, Scherer DC, Beilhack GF, Shizuru JA, Weissman IL (2003), Biology of hematopoietic stem cells and progenitors: implications for clinical application, Annu. Rev. Immunol. 21:759 806.

Marcacci M, Kon E, Zaffagnini S, Giardino R, Rocca M, Corsi A, Benvenuti A, Bianco P, Quarto R, Martin I, Muraglia A, Cancedda R (1999a), Reconstruction of extensive long bone defects in sheep using porous hydroxyapatite sponges, Calcif. Tissue Int. 64:83 90.

Marcacci M, Kon E, Zaffagnini S, Visani A (1999b), Use of autologous grafts for reconstruction of osteochondral defects of the knee, Orthopedics 22:595 600.
Noll T, Jelinek N, Schmid S, Biselli M, Wandrey C (2002), Cultivation of hematopoie tic stem and progenitor cells: biochemical engineering aspects, Adv. Biochem. Eng. Biotechnol. 74:111 128.
Orkin SH (2000), Diversification of haematopoietic stem cells to specific lineages, Nat. Rev. Genet. 1:57 64.
Shizuru JA, Negrin RS, Weissman IL (2005), Hematopoietic stem and progenitor cells: clinical and preclinical regeneration of the hematolymphoid system, Annu. Rev. Med. 56:509 538.
Sorrentino BP (2004), Clinical strategies for expansion of haematopoietic stem cells, Nat. Rev. Immunol. 4:878 888.
Spradling A, Drummond Barbosa D, Kai T (2001), Stem cells find their niche, Nature 414:98 104.
Taichman RS (2005), Blood and bone: two tissues whose fates are intertwined to create the hematopoietic stem cell niche, Blood 105:2631 2639.
Zhang J, Niu C, Ye L, Huang H, He X, Tong WG, Ross J, Haug J, Johnson T, Feng JQ, Harris S, Wiedemann LM, Mishina Y, Li L (2003), Identification of the haematopoietic stem cell niche and control of the niche size, Nature 425:836 841.

Gene therapy

21

Célio Lopes Silva, Karla de Melo Lima,
Sandra Aparecida dos Santos, and José Maciel Rodrigues Jr

21.1 Introduction

Gene therapy means the introduction of a gene into a cell to correct a defect. This is intended to slow the progression of a disease and consequently improve an individual's quality of life. The availability of the human genome map in 2000 was the first major step to enhance the potential impact of gene therapy as well as cell therapy and tissue engineering (Romano et al 2000). However, establishing these technologies requires *ex vivo* cell cultivation, and one of the challenges is to adapt the culture from the laboratory to a clinically relevant scale. Thus, the development of animal cell culture technology has been essential for advances in gene therapy.

21.2 Gene therapy

With the advent of new knowledge in cell and molecular biology, it has become evident that the causes of many human diseases are related to changes in genes. Genes represent the starting point for all events that subsequently lead to changes in expression of proteins in cells. Protein function determines, in turn, the cell phenotype and cell function. When genes are changed in such a way that the proteins encoded by them are unable to perform their normal function, a genetic disorder may occur. The cumulative effect of events triggered by atypical or defective genes in an organ leads to the disease phenotype. Gene therapy is a technique used to correct genetic defects that may cause the development of diseases, and consists of blocking an undesirably activated gene, or correcting or replacing the defective gene with a normal gene (*Figure 21.1*). The diseases primarily targeted by human gene therapy are monogenic, that is, those caused by defects in a single gene (Anderson, 1998). Since the 1990s, the development of DNA vaccines has led to the concept of gene therapy being extended to prophylaxis and treatment of infectious diseases (Brunnell and Morgan, 1998).

The first step in gene therapy is to identify the gene causing the problem. Then the appropriate genetic material is introduced into the target cells, with the aim of correcting the problem. Two main strategies are used: (i) *in vivo* therapy, in which the genetic material is introduced directly into the individual by a systemic route, or when possible, at a specific site; (ii) *ex vivo* therapy, in which the target cells are treated with

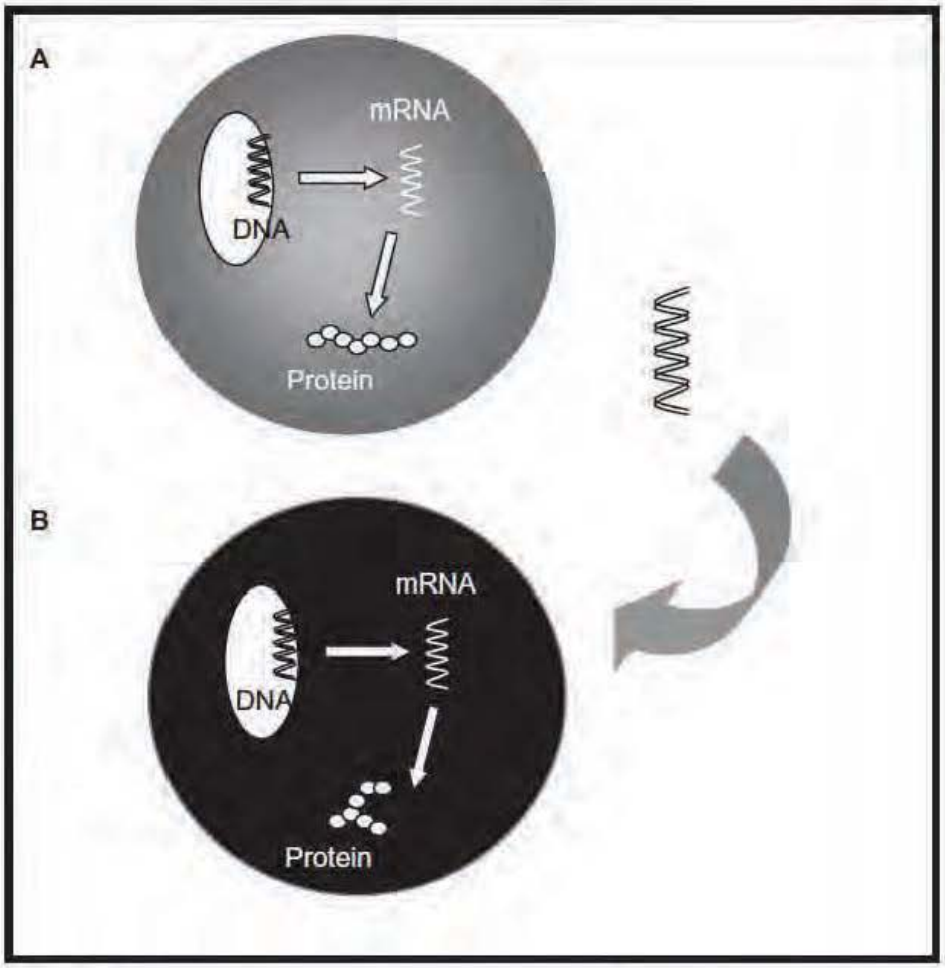


Figure 21.1

In a normal cell, genes are transcribed into messenger RNA (mRNA), and thereafter, translated into proteins with specific functions (A). When the gene responsible for encoding a protein has a defect, this may cause the translation of a defective protein, unable to perform its function and leading to a disease (B). Gene therapy aims to rectify the defect, by blocking, correcting, or replacing the gene.

the gene product outside the individual. The cells are expanded and then transferred back to the patient (*Figure 21.2*).

The key to successful *in vivo* gene therapy is the transfer of the genetic material to the target cells or tissues and the effective expression of the gene in question. However, this is a complex process, requiring several barriers to be overcome. *Ex vivo* gene therapy involves the transfer of genes to cells maintained in culture and their subsequent transplantation to the target tissue. The cells to be transfected must be available in large amounts, have a long life after transfer to the host, express the gene of interest for a long time period, and not trigger any kind of immune response. The advantages of *ex vivo* therapy include the possibility of characterizing the modified cell population before transfer. This makes it easier to introduce the genetic material into the cells and it can be insured that the clonal population of cells expresses a high amount of the product encoded by the transgene before transfer back into the patient.

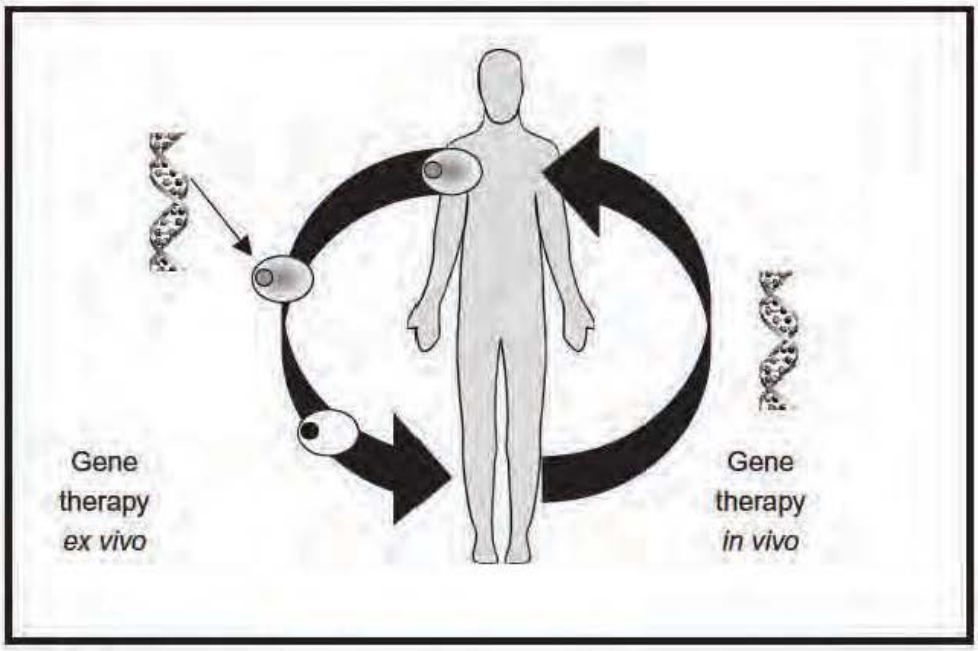


Figure 21.2

Strategies applied to gene therapy. *Ex vivo* the target cells are treated with the gene product outside the individual, expanded by cell culture, and then re introduced into the patient; or *in vivo* the genetic material is introduced directly into the individual by a systemic route, or, whenever possible, site specifically.

21.3 Vectors used in gene therapy

The development of vectors applicable in gene therapy has been the object of many studies. The vectors that have been developed can be divided into two categories: synthetic and viral vectors. Synthetic vectors depend on direct transfer of the genetic information into the target cell, and include plasmid DNA, naked DNA or DNA associated with some release system. Some may be applied as DNA vaccines (Ferber, 2001; Niidome and Huang, 2002). The viral vectors use the capacity of viruses for infection to introduce the genetic material into the target cells, and are most widely used in preclinical and clinical protocols for therapies in which a highly stable transfection is desired. Both systems have advantages and disadvantages, which must be considered depending on the intended therapy (Cristiano, 1998).

21.3.1 Viral vectors

The concept of viral vectors is based on the innate capacity of viruses to release their genetic material into infected cells. However, the biggest concern in using viruses as gene vectors for therapy is that viral infection may lead to undesired effects such as host tissue destruction. This destruction may be caused by the induction of genes whose products are harmful to the host cell, or by the insertion of the viral genetic material into the host cell genome, leading to pathogeneses, which could include tumor-

igenesis. Other disadvantages include the limitation on the size of genes that viruses can carry, the difficulty of standardizing the production of viral particles by cell culture, and the higher cost when compared with plasmid DNA production.

The basis for the use of viruses as gene vectors is the possibility of separating the components required for replication from those causing diseases (Hendrie and Russel, 2005). As discussed in Chapters 3 and 18, some initial information is required:

(i) identification of the viral sequence required for replication;
(ii) viral particle construction;
(iii) viral genome packing;
(iv) release of the heterologous gene into the host cell.

Among the viruses widely used for gene therapy are those used to obtain heterologous proteins from animal cells, particularly RNA viruses (retrovirus and lentivirus) and DNA viruses (adenovirus, adeno-associated viruses, and herpesvirus). *Table 21.1* summarizes the advantages and disadvantages of each virus as a carrier in gene therapy.

Adenoviruses are widely distributed in nature, and infect birds and many mammals, including man. They cause upper respiratory tract infection, in addition to other infections. Adenoviruses are non-enveloped viruses with a linear genome composed of double-stranded DNA. Subtypes 2 or 5 from subgroup C are predominantly used as vectors in gene therapy. During viral propagation, episomal replication occurs. This means that the virus genome does not integrate to the host genome, thus eliminating the risks of mutagenesis due to genomic insertion. The adenovirus genome is about 35 kb, from which 30 kb may be replaced with the transgene. Adenoviral vectors are highly effective upon transduction of target cells, and may be produced in high titre.

Adeno-associated viruses (AVVs) are non-pathogenic human parvoviruses. The application of these viruses depends on an auxiliary virus, usually an adenovirus. AVVs are able to infect dividing or non-dividing

Table 21.1 Advantages and disadvantages of use of different viral vectors in gene therapy

Viral vectors	Advantages	Disadvantages
Adenovirus	Infection of non dividing cells Attaining high titre in culture	Highly immunogenic Short term expression
AVV	Infection of non dividing cells Infection of different cell types	Limited integration Short term expression
HSV	Infection of different cell types	Attain low titre in culture Cytotoxicity Short term expression
Lentivirus	Attain high titre in culture Permanent infection of non dividing cells	May cause undesirable effects
Retrovirus	Integration of viral DNA in the host genome	Infection of dividing cells only

AVV, adeno associated virus; HSV, herpes simplex virus.

cells and, in the absence of an auxiliary virus, they may integrate into a specific site in the host genome. The AVV genome is composed of a single-stranded DNA molecule. The transgene contained in an AVV may not exceed 4.7 kb (Zaiss and Muruve, 2005).

Herpes simplex type 1 virus (HSV-1) is a human neurotropic virus, used as a vector for gene transfer in the nervous system. The HSV genome is linear, composed of a double-stranded, 152 kb DNA molecule, and able to contain 40–50 kb of a transgene.

Retroviruses represent a large family of enveloped RNA viruses. Endogenous retroviruses have been found in all vertebrate species. The viral particle contains two copies of viral genomic RNA in a conic nucleus. The viral RNA contains three essential genes, *gag*, *pol*, and *env*. The *gag* gene encodes the capsid, matrix, and nucleocapsid proteins, which are generated by proteolytic cleavage of the precursor protein *gag*. The *pol* gene encodes the viral enzymes: protease, reverse transcriptase, and integrase. The e*nv* gene encodes the envelope glycoproteins that mediate the virus entry in the cell. After connecting with the receptors on the cell surface, the capsid containing the genomic RNA enters the cell by fusing with the membrane. Thereafter, the genomic RNA is converted into double-stranded DNA by viral reverse transcriptase. The DNA is associated with the viral proteins in a complex that is translocated to the nucleus. An integrase enzyme mediates its integration into the host genome (Morizono and Chen, 2005). Retroviruses are the most widely used in gene therapy to date and have the advantage of promoting stable transfection, because the virus genome is inserted into the host cell genome. The disadvantages are that this insertion occurs randomly, which may modify cell genes in a harmful way, and that its particle is fragile, making handling and attainment of high titre difficult. Additionally, they infect only dividing cells (Nardi and Ventura, 2004).

Lentiviruses are a subclass of retroviruses expressing a complex that controls important nuclear functions in the infected cells. Lentiviruses are able to replicate in non-dividing cells, resulting in infection lasting the whole life of the host (Quinonez and Sutton, 2002).

21.3.2 Synthetic vectors: plasmid DNA

The advantages of using plasmid DNA as a gene vector are its capacity to carry a large amount of genetic material, the absence of risk of infection or mutagenesis, and easy large-scale production from cultivation of bacterial cells (Brown *et al.*, 2001). The plasmid is a circular DNA, which replicates independently from the cell chromosome. The plasmid used in gene therapy should have the following properties:

(i) replication origin in bacteria, to allow efficient replication of plasmids reaching hundreds of copies per cell;
(ii) a selection gene, which usually provides resistance to an antibiotic, to allow selection of clones carrying the plasmid;
(iii) a multiple cloning site, which may be cleaved with restriction enzymes, allowing insertion of the gene of interest;

(iv) a promoter, usually a viral promoter, which allows the transcription of the gene of interest in eukaryotic cells.

After the gene of interest is cloned into the plasmid, it is transferred to a host bacterium, usually *Escherichia coli*, by the process of bacterial transformation, to produce plasmids on a large scale and to obtain a sufficient amount of DNA for therapeutic use.

Carrier systems for plasmid DNA

Plasmid DNA may be administered to several animal species, including humans, by several routes and schedules. In addition to intramuscular injection, it may be administered orally, intranasally (as an airspray), or by an intradermal route, by bombing gold microparticles covered with the genetic material (Lima *et al.*, 2003a). Although plasmid intramuscular injection is a simple and widely used technique, there are some problems, such as the presence of enzymes (nucleases) able to degrade the plasmid DNA, making it ineffective. Another limitation is the size of the DNA molecule and its superficial molecular charge, which limits its penetration into the target cell. A prerequisite for use of DNA as a vaccine or gene therapy is that the nucleic acid is released effectively in the target cell. An estimation is that only one in every 1000 plasmid molecules administered is able to reach the nucleus and express the message for the desired protein synthesis, meaning that a treatment usually requires the administration of high plasmid DNA doses, of possibly several hundred micrograms up to milligrams (Friedman, 1997).

Several investigators have shown that intramuscular injection requires up to 100 times higher the amount of plasmid to generate an expression equivalent to one produced by DNA carrying systems, as discussed in the next few paragraphs. In contrast, with adsorption or encapsulation techniques in non-viable systems, the plasmid release may occur preferentially in the intracellular environment, avoiding functional plasmid degradation. Among the strategies used, we will mention the use of biobalistics, liposomes, lipoplexes, and polyplexes, in addition to the use of biodegradable polymeric microparticles.

Bioballistics or ''gene gun'' consists of transfecting individual cells, using DNA adsorbed onto gold particles (0.6–2 μm in diameter). For the transfection, the particles are placed in a device known as a gene gun (*Figure 21.3*), which, by means of an acceleration process using helium gas discharge under high pressure, is projected at an individual's skin, enabling the particles to reach the epidermis (Haynes *et al.*, 1996). Transfection using a gene gun requires a 100 times lower the amount of plasmid to generate an expression corresponding to one produced by intramuscular administration (Barry and Johnston, 1997), because bioballistics enables the release of the plasmid inside the cells, avoiding its degradation. This technique of introducing a vector by bioballistics, although effective for several transfection procedures, requires a specialized device for its use. However, promising clinical results have motivated companies to invest in improving this technology.

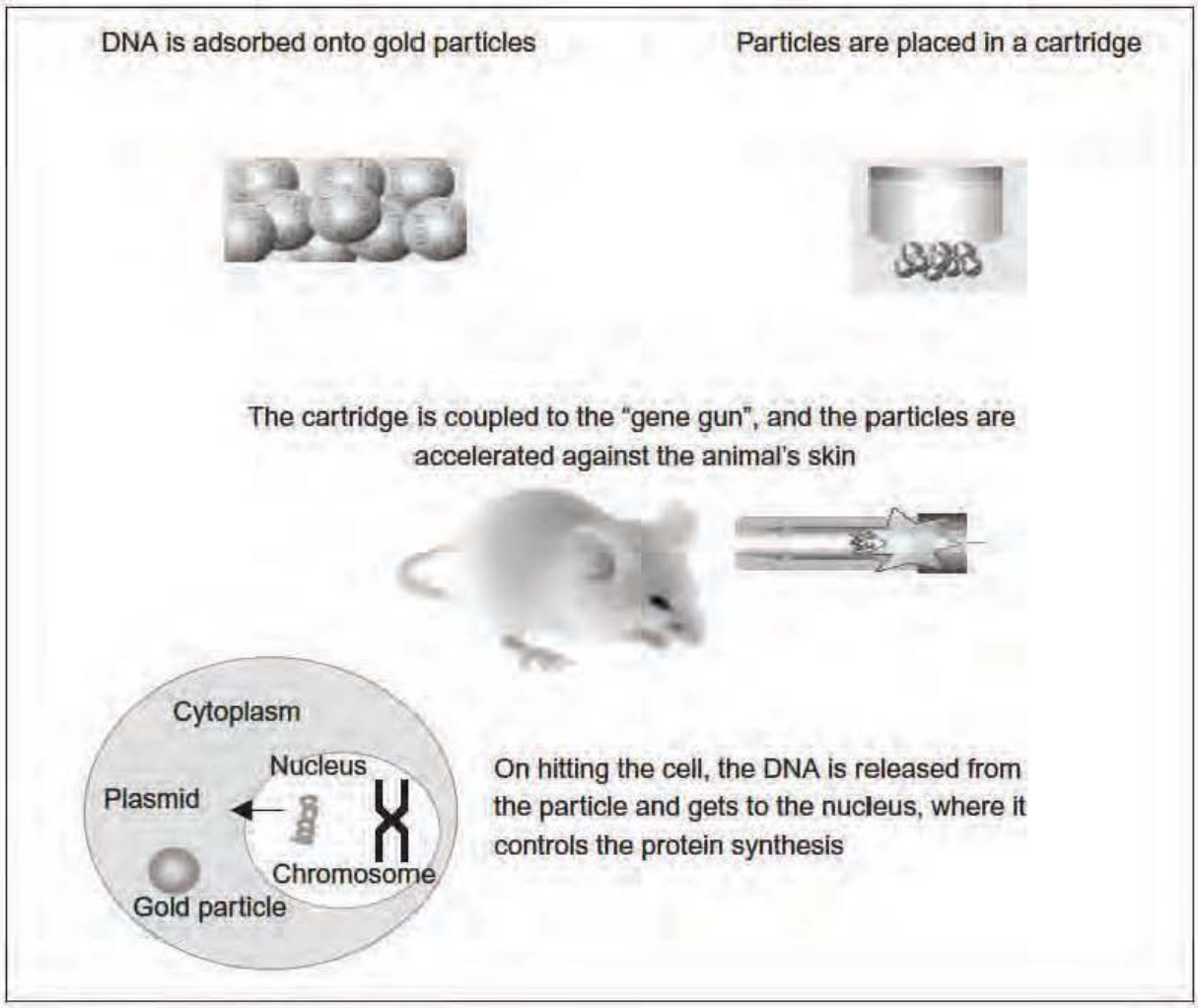


Figure 21.3

Use of bioballistics as a plasmid DNA carrier.

An alternative method for introducing a plasmid DNA vector is to use liposomes. As they are composed of aqueous vesicles surrounded by a phospholipid bilayer, liposomes allow encapsulation and transportation of many substances, both hydrophilic and lipophilic, along with the plasmid. Liposomes also allow molecules such as antibodies, proteins, and sugars to be incorporated into their surface, to target them to specific sites. Due to the structural versatility shown by these systems, the chances of effective transfection may be increased by changes in the lipid composition, which may alter the superficial charge or the vesicle size (Bramwell and Perrie, 2005). Cationic liposomes have wide applications in gene transfection.

Lipoplexes and polyplexes are DNA–cationic molecular complexes, formed, respectively, by DNA interaction with lipids or polymers. The main property of these complexes is to allow easier passage of DNA through the cell plasma membrane, by means of two mechanisms: DNA charge neutralization and plasmid condensation, which reduces its size. Such complexes are formed by an excess of positive charges to neutralize DNA phosphate groups, resulting in transfecting particles with a net

positive charge. The effectiveness of this approach is associated with adsorptive endocytosis between the positive particle and the cell's negative surface. Once inside the cell, the complexes containing the DNA are released from the endocytotic vesicle and spread through the cytosol leading to the nucleus. However, this electrostatic adsorption may also result in low bioavailability *in vivo*, and the absence of cellular specificity, probably due to interactions with the cell surface proteoglycans and to polyanionic glycans present in the extracellular matrix (Mislick and Baldeschwieler, 1996). Thus, the use of this method may be limited by several factors: its variable plasma binding when the complexes are administered systemically; its association to macromolecules present in the extracellular matrix; the low efficacy of membrane penetration through endocytosis; and its passage from cytoplasm to nucleus.

Some charged polymers, such as polyethyleneimine (PEI) have a property known as proton sponge. When at an acidic pH, as in an endolysosomal compartment, PEI expands, which may cause the organelle it is in to break, resulting in DNA release into the cytosol. Thus, some complexes, in addition to allowing easier DNA penetration into the cell, may also make their traffic to the nucleus easier, and contribute to reduced plasmid degradation.

Recently, biodegradable polymeric spheres have been developed as an interesting strategy to be used in the transfection process (Lima *et al*, 2003b). These microspheres are composed of lactic and glycolic acid polyesters, and have the advantage of being biodegradable, with no adverse effects at the site of administration, if used by the parenteral route. In the organism, these polymers are hydrolyzed, and once degraded, they release lactic and glycolic acid, which are innocuous substrates for the organism (*Figure 21.4*). The potential of these systems as carriers is associated with:

(i) the protection of encapsulated plasmid, allowing a reduction in the amount to be used;

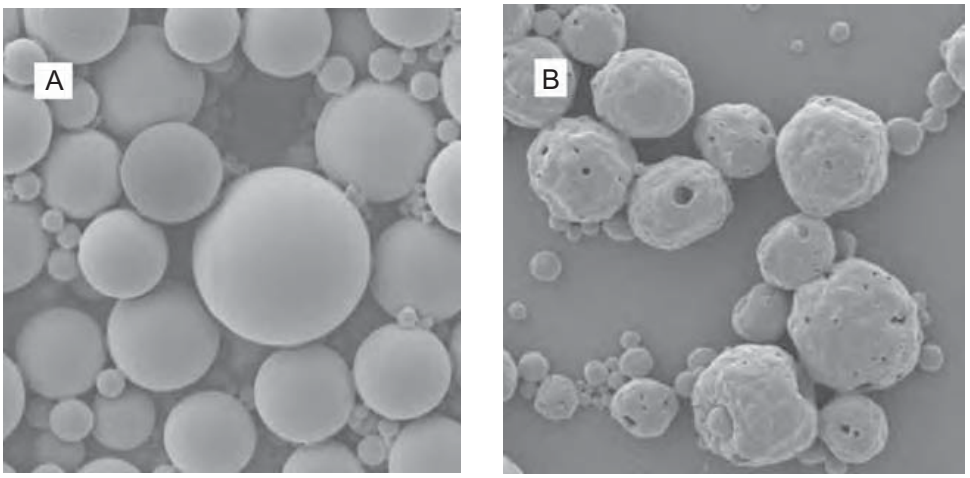


Figure 21.4

Electron scanning micrograph showing intact polymeric microspheres (A) or matrix degradation (B).

(ii) interaction with mononuclear phagocytic cells, since particles with a diameter under 10 μm are easily phagocytosed by macrophages, which could contribute to triggering the immune response, making them good vehicles to carry DNA;
(iii) the possibility of administering the plasmid by other routes, such as oral, nasal, and pulmonary;
(iv) easy administration;
(v) stability, once the spheres are stored as lyophilized powder, which may be reconstituted immediately before administration.

21.4 Principles of gene therapy

As shown in *Figure 21.5*, gene therapy is based on three basic principles: (i) replacement or correction of a gene for the purpose of generating products appropriate to cell or organ function; (ii) the introduction of a heterologous gene into the target cell, leading it to produce something that is not innate; (iii) inactivation of a gene causing a cell dysfunction.

21.4.1 Replacement or correction of a mutant gene

The replacement of an abnormal gene with a normal copy of the same gene may restore the cell capacity to produce functional proteins, contributing to cell homeostasis (*Figure 21.5B*). This type of gene therapy is one of the

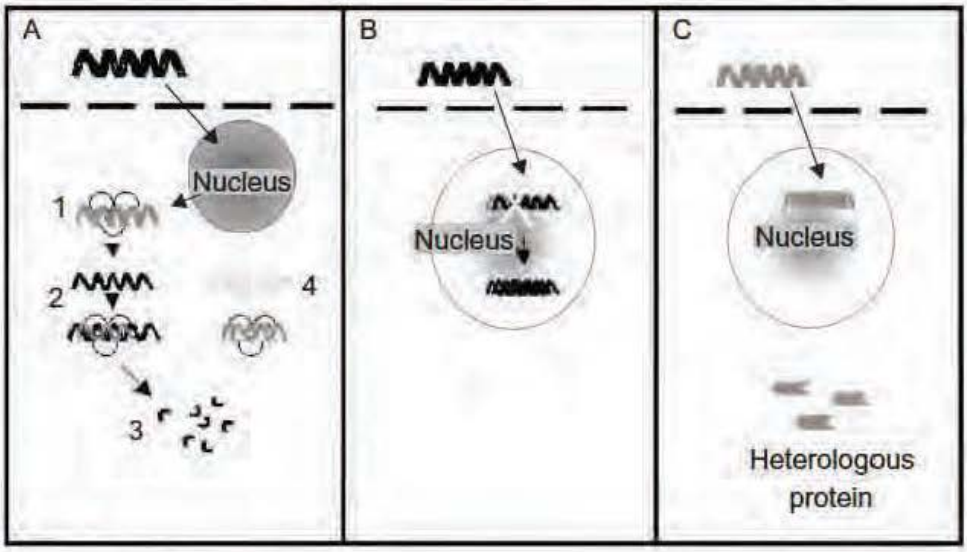


Figure 21.5

Basic principles of gene therapy. (A) Gene inactivation by interference RNA (iRNA). The DNA encoding the iRNA is introduced in the cell. The iRNA forms the silencing complex with proteins present in the cytoplasm (1). The mRNA generated by the changed gene is homologous to the iRNA and connects to the silencing complex (2), being destroyed by it (3). The mRNA generated by normal genes is not affected by the iRNA (4). (B) Gene replacement. The defective gene is replaced with a normal copy of the same gene. (C) Introduction of a heterologous gene. The gene encoding a protein from another organism is introduced into the target cell, allowing it to produce the heterologous protein.

most widely used, and may be applied to different types of diseases. Usually, viral vectors are used in this kind of therapy, since in most cases long-lasting transgene expression is required.

21.4.2 Introduction of a heterologous gene

This technique is especially used in DNA vaccine protocols or therapies for infectious diseases, where the objective is to trigger an immune response against a specific antigen. In this case, the gene encoding an antigenic protein of a pathogen is identified and inserted into an expression vector, which is then transferred to the target cell to make it start producing the heterologous protein in question (*Figure 21.5c*). In this case, the plasmid DNA-based synthetic vectors are the most commonly used, due to safety reasons and because these are generally prophylactic interventions.

21.4.3 Gene inactivation

When the expression of the product of an abnormal gene leads to cell imbalance, and the gene expression is not fundamental for the cell or for the individual, one of the therapeutic alternatives is its inactivation. The inactivation of a gene may be performed at the DNA, messenger RNA, or protein level. In several organisms, the introduction of double-stranded RNA has proved to be a powerful tool to suppress gene expression, in a process known as interference RNA (iRNA), and has been largely used in gene therapy research. The use of iRNA is a process in which the double-stranded RNA induces degradation of the homologous messenger RNA (*Figure 21.5A*). However, in most mammalian cells, this causes a strong cytotoxic response, which may be controlled by using synthetic iRNA able to mediate a highly specific suppression of the target gene. Although effective, the silencing caused by the synthetic iRNA is transient, limiting its application. To mitigate this problem, plasmid and viral vectors have been created, to produce the iRNA inside the cells.

21.5 Gene therapy and clinical studies

From 1989 up to the present, over 500 clinical studies of gene therapy have already been approved and are being conducted worldwide, as shown in *Figure 21.6*. About 70% of these studies are intended for cancer treatment, and most (97%) are conducted in Phase I and II in terminal patients. About 3% are conducted in Phases II/III or III. So far, only a single product (Gendicine®) was approved in 2003 for marketing, and is currently in assessment Phase IV. Although it is not part of that statistic, in Brazil there is only one Phase I study, started in 2004 and currently in progress, to assess the effectiveness of plasmid DNA encoding the thermal shock protein hsp65 of *Mycobacterium leprae* for head and neck epidermal cancer treatment (non-published data). *Table 21.2* shows the number of approved clinical studies and the study phases.

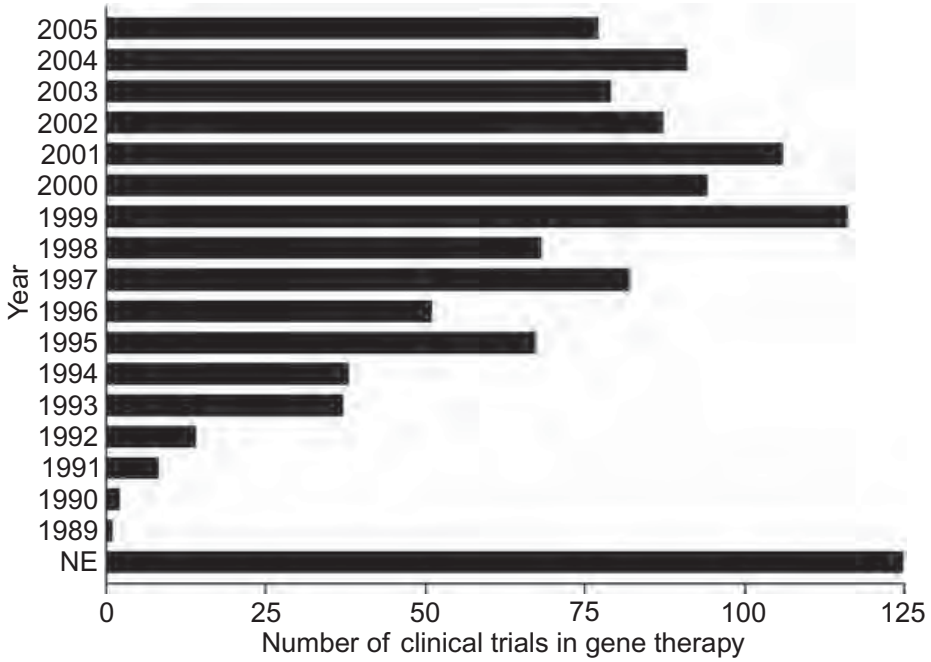


Figure 21.6

Number of clinical trials in gene therapy approved worldwide between 1989 and 2005 (www.wiley.co.uk/genmed/clinical). NE, not established.

Table 21.2 Gene therapy protocols classified by clinical study phase

Phase	Gene therapy clinical studies	
	Number	%
Phase I	714	62.4
Phase I/II	234	20.4
Phase II	161	14.1
Phase II/III	12	1
Phase III	24	2.1
Total	1145	100

Adapted from *Journal of Gene Medicine* 2006 (www.wiley.co.uk/genmed/clinical).

Table 21.3 Gene therapy clinical trials classified by the type of disease

Indications	Gene therapy clinical trials	
	Number	%
Cancer	762	66.6
Marking genes	52	4.5
Healthy volunteers	19	1.7
Infectious diseases	75	6.6
Monogenic diseases	100	8.7
Others	37	3.2
Vascular diseases	100	8.7
Total	1145	100

Adapted from *Journal of Gene Medicine* 2006 (www.wiley.co.uk/genmed/clinical).

Table 21.3 shows the clinical studies that have been conducted worldwide, with their different applications, showing that therapies intended for monogenic disease treatment are the second most assessed group, after therapies for tumor treatment. The most used vectors in gene therapy clinical studies are viral vectors (68%), and among those, retroviruses and adenoviruses are the viruses of choice. Synthetic vectors were used in 25% of the studies performed, and about 16% correspond to the use of naked plasmid DNA (*Table 21.4*).

Although many gene therapy protocols have been approved for clinical trial in humans, gene therapy safety has been questioned, due to two adverse events. The first event involved the death, in 1999, of a young man who underwent experimental therapy in the United States. This patient had a rare genetic disorder known as ornithine transcarbamylase (OTC) deficiency, which affects the individual's capacity to eliminate ammonia, a toxic product resulting from protein metabolism. The second event occurred in 2002, when it was announced that children treated for severe combined immunodeficiency (SCID) syndrome had signs of leukemia after receiving the treatment (Hacein-Bey-Abina *et al.*, 2003). This syndrome, also known as ''bubble boy disease,'' is caused by a single mutated

Table 21.4 Vectors used in gene therapy clinical trials

Vector	Gene therapy clinical trials	
	Number	%
Adeno associated viruses	38	3.32
Adenovirus	287	25.07
Gene gun	5	0.44
Flavivirus	5	0.44
Herpes simplex virus	38	3.32
Lentivirus	5	0.44
Lipofection	95	8.30
Listeria monocytogenes	1	0.09
Measles virus	2	0.17
Naked plasmid DNA	192	16.77
Naked plasmid DNA + adenovirus	1	0.09
Newcastle disease virus	1	0.09
Poliovirus	1	0.09
Recombinant Poxvirus	1	0.09
Poxvirus	59	5.15
Retrovirus	276	24.10
Transference RNA	14	1.22
Saccharomyces cerevisiae	2	0.17
Salmonella typhimurium	2	0.17
Semliki Forest virus	1	0.09
Simian 40 virus	1	0.09
Vaccinia virus	51	4.45
Adenovirus + retrovirus	3	0.26
Poxvirus + vaccinia virus	21	1.83
Not commented	43	3.76
Total	1145	100

Adapted from *Journal of Gene Medicine* 2006 (www.wiley.co.uk/genmed/clinical).

gene, and forces the individual to live under sterile conditions, since he/she does not have an effective defense against infections. Tumor cells were found in the blood of the patients following the gene therapy, containing an intact copy of the retroviral vector that had integrated near or in the LMO2 gene. Although the exact mechanism by which the LMO2 gene activation may have been responsible for the origin of leukemic cells is not clear, discussions led to debates about the safety of using viral gene vectors. These questions are reflected in the sharp fall in the number of new clinical trial protocols approved recently, as shown in *Figure 21.6*.

Other causes for failure of gene therapy in clinical trials up to now include: short-lasting therapeutic effect; triggering of host's immune response mainly against viral vectors; problems with viral vectors, such as treatment of patients with SCID, which caused the development of complications, leading to death; multigenic disorders, such as heart conditions, Alzheimer's disease, arthritis, and diabetes.

21.5.1 The first gene therapy product

Despite all these questions related to the safety of gene therapy, efforts are still being made to eliminate the existing problems. These efforts reached their highest point for the approval of the first product for gene therapy, which is based on an adenoviral vector. It is Gendicine®, a medication produced by the Chinese company, Shenzhen Sibiono GeneTech. The medication is intended for head and neck carcinoma treatment, and was approved for marketing in 2003 by China's regulatory agency (China's State Food and Drug Administration – SFDA). Recombinant adenovirus, in the form of 90 nm particles, contain the tumor-suppressing gene p53. p53 gene is mutated in about 50–70% of human tumors. The mutant genes are not necessarily inactive, but may have oncogenic functions that contribute to tumor genesis. Proteins originating from the mutant gene are also associated with over-regulation of a multidrug-resistant gene, which results in resistance to several chemotherapeutics. The exogenous introduction of gene p53 and subsequent expression of protein p53 leads to tumor control and elimination. A synergistic effect may also be obtained when the gene therapy is associated with radiotherapy and chemotherapy.

Gendicine® is produced in SBN cells, a cell lineage subcloned from human embryonic renal cells, lineage 239 (HEK-293). As these are adherent cells, culture was at first assessed in roller bottles, but this did not prove satisfactory for production. As an alternative, a parallel plate reactor (CellCube®) was used. In this kind of system, the production obtained was low (about 4.9×10^9 viral particles/cm^2). Subsequently, the development of a perfusion culture process in packed-bed bioreactors using disks (Fibra-Cel™ Disks) as support provided a 15-fold higher production, compared with the CellCube®. In addition, a batch culture using suspended cells and fetal bovine serum-free medium is being developed. The most important point in the production process was the preparation of reference and working cell banks. After production of adenoviral vectors in bioreactors, they are submitted to clarification and ultrafiltration, and finally purified in automated systems. After the whole downstream process, the recovery rate of viral particles is about 65%. The purified and

formulated product is dispensed in sterile glass bottles and stored frozen, up to the time of use.

21.6 Perspectives

Gene therapy is still new, and its scientific base will be established with further preclinical and clinical tests. Although many patients are currently being evaluated, several questions are posed about vector safety. The advent of non-viable vectors with biodegradable substances and construction of mimetic systems will be of great value for solving the problems associated with the viral vectors. Nanotechnology tools, as well as new cell visualization and characterization tools, will allow the advent of safer transfection techniques. As there are several disciplines involved, advances in gene therapy depend on different technologies, which may be obtained only by multidisciplinary research.

The collection of toxicological data is important to allow the clarification of the level of risk of different classes of viral vectors. The approval of the first medication intended for gene therapy opens new perspectives to obtain such essential data at a critical time for discussion about the safety of these vectors. Such data are very valuable for regulating agencies. It is important to emphasize that investigators and businessmen interested in this area are responsible for ensuring that the patients' health and well-being are the paramount objectives, and strict adherence to currently available rules and guidelines is required. Additionally, the increasing availability of new data will form the basis of future guidelines for production, marketing, clinical trials, and safety for these products.

Detailed knowledge of the human genome, which has been achieved in the last few years, provide a rapid development tool and targets for gene therapy. A great impact is expected on human healthcare with this new knowledge, creating high expectations concerning the genesis of new products intended for the treatment of infectious diseases and tumors, which do not have alternative treatments.

On the other hand, from the technological point of view, several barriers have already been overcome, allowing production of viral or plasmid DNA vectors at a cost that enables clinical application. Safe manufacturing processes are already available, and the production capacity for these systems is easily accessible worldwide.

Thus, it is possible to conclude that this therapy, still in its early phases, will cause a great impact on human health in the next few decades. Furthermore, the scale-up of biomanufacture of products for clinical use from technologies for animal cell cultures will be an important step in the application of gene therapy,

References

Anderson WF (1998), Human gene therapy, Nature 392(6679 Suppl):25 30.

Barry MA, Johnston SA (1997), Biological features of genetic immunization, Vaccine 15:788 791.

Bramwell VW, Perrie Y (2005), Particulate delivery systems for vaccines, Crit. Rev. Ther. Drug. Carrier Syst. 22:151 214.

Brown MD, Schatzlein AG, Uchegbu IF (2001), Gene delivery with synthetic (non viral) carriers, Int. J. Pharm. 229:1 21.
Brunnell A, Morgan R (1998), Gene therapy for infectious diseases, Clin. Microbiol. Rev. 11:42 56.
Cristiano, RJ (1998), Viral and non viral vectors for cancer gene therapy, Anticancer Res. 18:3241 3245.
Ferber D (2001), Gene therapy. Safer and virus free?, Science 294:1638 1642.
Friedman T (1997), Overcoming the obstacles to gene therapy, Sci. Am. 97:80 85.
Hacein Bey Abina S, von Kalle C, Schimidt M, Le Deist F, Wulffraat N, McIntyre E, Radford I, Villeval JL, Fraser C, Cavazzana Calvo M, Fischer A (2003), A serious adverse event after successful gene therapy for X linked severe combined immunodeficiency, N. Engl. J. Med. 348:255 256.
Haynes JR, McCabe DE, Swain WF, Widera G, Fuller JT (1996), Particle mediated nucleic acid immunization, J. Biotechnol. 44:37 42.
Hendrie PC, Russell DW (2005), Gene targeting with viral vectors, Mol. Ther. 12: 9 17.
Journal of Gene Medicine (2005). Available at: www.wiley.co.uk/genmed/clinical (accessed May 2006).
Lima KM, dos Santos SA, Santos RR, Brandao IT, Rodrigues JM Jr, Silva CL (2003a), Efficacy of DNA hsp65 vaccination for tuberculosis varies with method of DNA introduction *in vivo*, Vaccine 22:49 56.
Lima KM, dos Santos SA, Lima VM, Coelho Castelo AA, Rodrigues JM Jr, Silva CL (2003b), Single dose of a vaccine based on DNA encoding mycobacterial hsp65 protein plus TDM loaded PLGA microspheres protects mice against a virulent strain of *Mycobacterium tuberculosis*, Gene Ther. 10:678 685.
Mislick KA, Baldeschwieler JD (1996), Evidence for the role of proteoglycans in cation mediated gene transfer, Proc. Natl Acad. Sci. U S A 93:12349 12354.
Morizono K, Chen IS (2005), Targeted gene delivery by intravenous injection of retroviral vectors, Cell Cycle 4:231 237.
Nardi NB, Ventura AM (2004), Terapia gênica, In: Mir L (Ed.), Genômica, Editora Atheneu, São Paulo, pp. 625 542.
Niidome T, Huang L (2002), Gene therapy progress and prospects: non viral vectors, Gene Ther. 9:1647 1652.
Quinonez R, Sutton RE (2002), Lentiviral vectors for gene delivery into cells, DNA Cell Biol. 21:937 951.
Romano G, Michell P, Pacilio C, Giordano A (2000), Latest development in gene transfer technology: achievements, perspectives and controversies over therapeutic applications, Stem Cells 18:19 39.
Zaiss AK, Muruve DA (2005), Immune responses to adeno associated virus vectors, Curr. Gene Ther. 5:323 331.

Appendix

Case study: Mathematical modeling of the growth of a hybridoma

From a set of three experiments (initial conditions in *Table A1*) conducted in batch mode, employing a secreting monoclonal anti-TNP (trinitrophenyl) hybridoma, various phenomena were observed and detailed below.

(i) The specific growth rate is limited by glutamine and inhibited by secreted ammonium and lactate (Equation A.1).
(ii) The specific death rate is inhibited by glutamine and limited by lactate and ammonium (Equation A.2).
(iii) The viable cell production rate is equal to the difference between the growth rate of viable cells and the death rate (Equation A.3).
(iv) The glucose consumption rate is divided in cell growth, first equation term, and lactate formation (Equation A.5).
(v) The glutamine consumption rate is divided in a first order chemical decomposition, first equation term, viable cell formation, second equation term, and ammonium formation (Equation A.6).
(vi) The variation of monoclonal concentration is partially associated with cellular growth (Equation A.7).
(vii) The lactate production rate is partially associated with cell growth (Equation A.8).
(viii) The ammonium production rate is associated with cell growth (Equation A.9).

The proposed mathematical modeling to fit the experimental data is based on Bree *et al.* (1988), and is represented by a set of differential Equations A.1 to A.9, consisting of seven state variables (*Table A2*) and 20 fitted parameters (*Table A3*).

Table A1 Initial conditions for tests

Test	$X_{V,0}$ (cel/ml)	GLC_0 (g/L)	GLN_0 (g/L)	$NH^+_{4,0}$ (mg/L)	LAC_0 (g/L)
1		4.5	0.58		
2	1.0×10^5	5.6	1.45	0.0	0.0
3		11.3	2.90		

Adapted from Lee, 2003.

Table A2 Model state variables

X_v	Viable cells concentration (cel/ml)
X_d	Non viable concentration (cel/ml)
GLC	Glucose concentration (g/L)
GLN	Glutamine concentration (mg/L)
mAb	Monoclonal antibody concentration (mg/L)
LAC	Lactate concentration (g/L)
NH_4^+	Ammonium concentration (mg/L)

Table A3 Fitted parameters

Parameters	Description	Fitted value
$\mu_{X,max}$	Maximum specific cell growth rate (h^{-1})	0.0630
k_{GLN}	Glutamine limitation constant for growth (mg/L)	2.65
k_{i,NH_4^+}	Ammonium inhibition constant for growth (mg/L)	65.73
$k_{i,LAC}$	Lactate inhibition constant for growth (g/L)	15.14
$k_{d,max}$	Maximum specific cell death rate (h^{-1})	0.0763
$k^d_{NH_4^+}$	Ammonium limitation constant for death (mg/L)	21.86
k^d_{LAC}	Lactate limitation constant for death (g/L)	6.114
$k^d_{i,GLN}$	Glutamine inhibition constant for death (mg/L)	778.2
$Y_{Xv/GLC}$	Glucose to viable cells yield coefficient (10^6 cell/10^{-3} g)	1.16
$Y_{LAC/GLC}$	Glucose to lactate yield coefficient (g/g)	1.00
α_1	Constant (10^{-3} g/10^6 cell)	0.520
$Y_{X_v/GLN}$	Glutamine to viable cells yield coefficient (10^6 cell/10^{-3} g)	20.85
$Y_{NH_4^+/GLN}$	Glutamine to ammonium yield coefficient (mg/mg)	0.0210
β_1	Constant (10^3 mg/10^6 cell)	7.05
α_2	Constant (10^{-3} g/10^6 cell)	8.67
α_3	Constant (10^{-3} g/10^6cell.h)	1.15
α_4	Constant (10^{-3} mg/10^6 cell)	24.99
β	Parameter no associated to growth (10^{-3} g/10^6 cell.h)	0.389
β_2	Constant (10^{-3} g/10^6cell.h)	0.312×10^{-3}
K	First order constant for glutamine decomposition (h^{-1})	0.334×10^{-2}

$$\mu_X = \mu_{X,max} \cdot \left[\frac{GLN}{k_{GLN} + GLN}\right] \cdot \left[\frac{k_{i,NH_4^+}}{k_{i,NH_4} + NH_4^+}\right] \cdot \left[\frac{k_{i,LAC}}{k_{i,LAC} + LAC}\right] \quad (A.1)$$

$$k_d = k_{d,max} \cdot \left[\frac{NH_4^+}{k^d_{NH_4} + NH_4^+}\right] \cdot \left[\frac{LAC}{k^d_{LAC} + LAC}\right] \cdot \left[\frac{k^d_{i,GLN}}{k^d_{i,GLN} + GLN}\right] \quad (A.2)$$

$$\frac{dX_V}{dt} = \mu_X . X_V - k_d . X_V \quad (A.3)$$

$$\frac{dX_d}{dt} = k_d . X_V \quad (A.4)$$

$$\frac{dGLC}{dt} = -\frac{\mu_X.X_V}{Y_{X_V/GLC}} - \frac{\alpha_1.\mu_X.X_V}{Y_{LAC/GLC}} \quad (A.5)$$

$$\frac{dGLN}{dt} = -k.GLN - \frac{\mu_X.X_V}{Y_{X_v/GLN}} - \frac{\beta_1.\mu_X.X_v}{Y_{NH_4^+/GLN}} \quad (A.6)$$

$$\frac{dMAb}{dt} = \frac{\alpha_2.\mu_X.X_V}{Y_{MAb/Xv}} + \beta.X_V \quad (A.7)$$

$$\frac{dLAC}{dt} = \alpha_3.\mu_X.X_V + \beta_2.X_V \quad (A.8)$$

$$\frac{dNH_4^+}{dt} = \alpha_4.\mu_X.X_V \quad (A.9)$$

Figure A1 illustrates the model simulation results with the final fitted parameters of one of the experiment 2. The proposed model represents adequately the characteristic phenomena of the process.

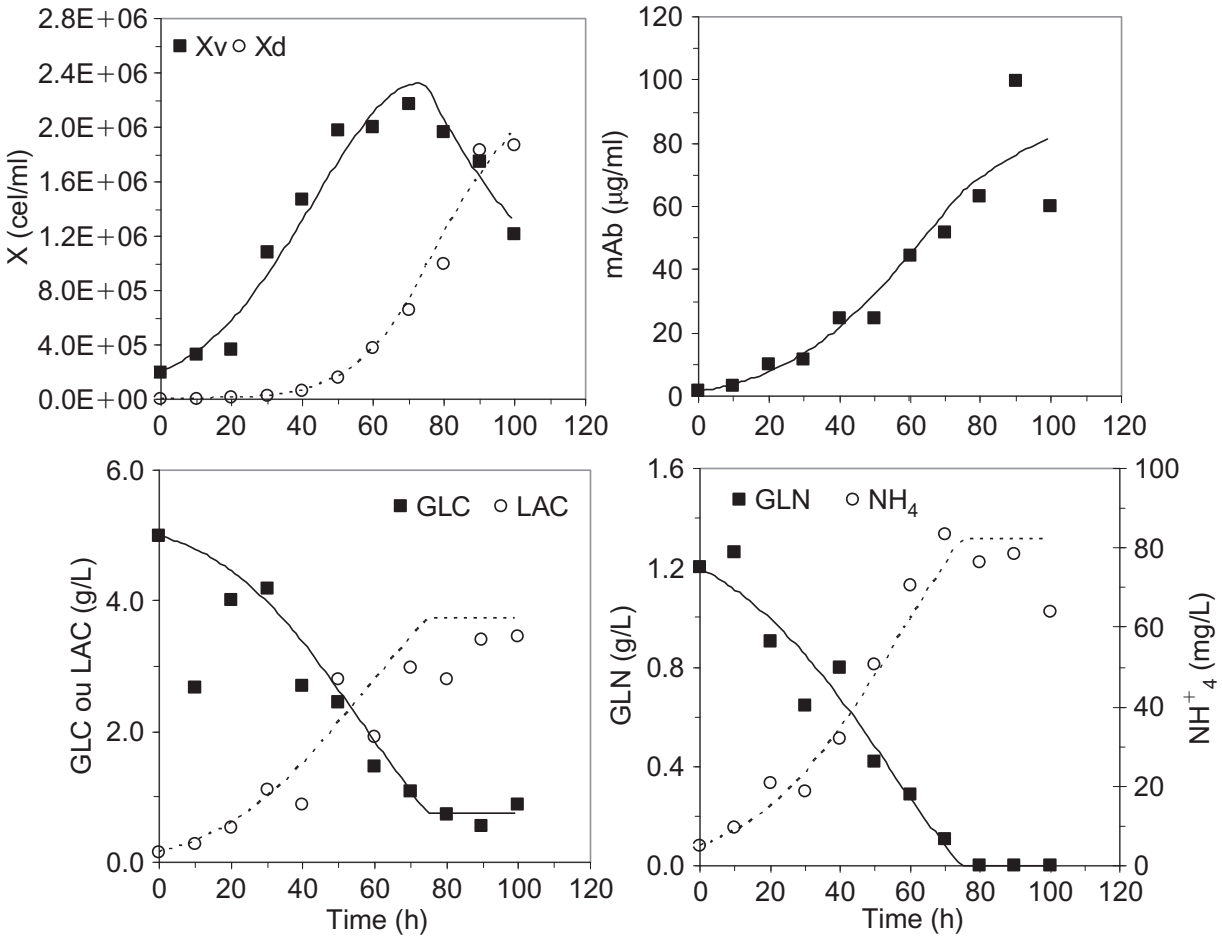


Figure A1

Experimental data simulation: (symbols) experimentals data; (line) model simulation.

Index

2-oxoglutarate 142
3T3 cells 4, 120

Abbokinase® 398
Acetyl CoA 79, 81–82, 88
Acetylation 134
Acoustic separators 290
Acridine orange 157, 158
Activase® 394, 402
Active transport 98, 100
Acute myocardial infarction 394, 398
Adeno-associated virus 492, 500, 503
Adenovirus 9, 31, 442, 452, 453, 492, 500, 501
Adherent cells 19–23, 27, 112, 118, 443, 444, 445
Adventitious agents (*see* Product contaminants)
Aeration (*see also* Oxygen supply) 26, 246, 261, 263, 265
Affinity chromatography 301, 314–319, 323, 335, 418
Affinity ligands 314–318
  Biospecific 316
  Matrix coupling 317–318
  Pseudo-biospecific 316
Aggregation of product 330, 331, 336, 337, 338
Agitation 246
Alanine 76, 79, 82, 85–91, 94–95, 99, 101, 112, 114, 123 184, 186, 201, 429
Alemtuzumab 3
Allosteric regulation 75, 89
Allotransplants 475–476
Alpha-ketoglutarate 79, 82, 84, 86, 88, 97, 101
Amadori re-arrangement 140
Amino acid 75–78, 80–82, 84–90, 112, 114, 116–117, 118, 120, 122, 123, 124, 130, 133–134, 140, 351, 316, 366, 425
  Sequence 331, 336–337, 339, 341, 423
Ammonia/Ammonium 76, 84–87, 91, 94–102, 116, 123, 138, 184, 186, 192, 200, 201, 203, 209, 214, 215, 428, 429, 500
Ammonium sulfate 302, 313
Amyloid 139
Anaplerotic reaction 82
Anchorage-dependent cells 20, 21
Anemia 392, 394
Angiogenesis 475
Anion exchangers 311–312
Annexin V 158
Antennarity 98–99, 133
Antibiotics 30, 114, 120
Antibodies 338, 339, 341, 343, 396, 399, 411–415
  Affinity 416, 419, 420, 422
  Antigenic determinant 409, 412, 413, 422
  Bi-functional 425
  Bi-specific 425
  Chimeric 420, 421
  Domains 411, 412, 419, 422, 423, 425
  Gene recombination 412, 413, 423, 427
  Half life 423, 425
  Humanized 420, 421
  Specificity 409, 410, 415, 419, 420, 425
  Variety 410, 412, 413
Antibody fragments 410, 420, 421, 422–425
  Diabodies 421, 425
  Tetrabodies 421, 425
  Triabodies 421, 425
Antigen 6, 316, 374, 380, 381, 383, 435, 444, 448, 450, 451, 455
Antioxidant enzymes 102
Apligraf® 8
Apoptosis 23, 27, 122, 124, 151, colour plate/ Fig. 7.3
  Molecular aspects 159
  Morphological characteristics 151, 156, 157, 160, 162, 168
  Control 171
  Definition 151
  Detection 155
  Induction 152
Apoptosome 167, 168, 169, 170, 171
Apoptotic pathways 160
  Intrinsic mitochondrial pathway 162
  Intrinsic ER stress-induced pathway 169
  Extrinsic death receptor-induced pathway 170
Aranesp® 394, 406
Arginine 79, 88–89, 112, 139, 142
Ascorbate 142
Asparagine 84–85, 87–90, 130, 138, 142
ATP 10, 16, 75–80, 83, 89, 92, 102
Autoimmune diseases 389, 391, 399, 400, 475
Avastin® 3, 396, 399, 419
Avonex® 3, 391, 394, 402

B lymphocyte 409, 410, 412, 413, 414, 416, 419, 420, 422, 482–483

Baculovirus 6, 32, 135, 136, 300, 448, 449, 450, 451,455, 459–470
Baculovirus expression vector system 6
Baculovirus infection cycle 461–462, 463, 464, 468
  Passage effect 467, 470
BAE-1 20
Balb/c 125 415
Basiliximab 3
Bcl-2 153, 154, 162, 164, 169, 171
Benefix® 3, 394, 399, 402
Betaferon® 391
Beta-hydroxyaspartate 130
Beta-oxidation 92
Betaseron® 391
Bevacizumab 3
Bexxar® 419
BHK cells 2, 3, 4, 6, 10, 20, 30, 55, 81, 83, 86–90, 93, 95–96, 99, 103, 130, 141, 120, 125, 443, 427, 429
Bioassays 330, 331, 341–345
Bioballistics 494–495
Biogenerics 366–367
Bioinsecticide 459–474
  Comparison to chemical pesticides 459–460
  Comparison to *in vivo* production 460
  Mechanism of action 460–463
  Virulence 460, 463, 466–467
Biopesticide (*see* Bioinsecticide)
Biopharmaceuticals 1, 2, 3, 6, 30, 122, 383, 388, 389, 394, 400, 402, 404
  Characterization 404
  Formulation and delivery 402
  Market 400
  Second-generation molecules 405
  Stability 402, 403
Bioreactors
  Air-lift bioreactors 225, 227
  Bioreactors for attached cell growth 225, 231
  Cell concentration ranges 254
  Classification of bioreactors 225
  Economic aspects 252
  Fluidized-bed bioreactors 225, 231
  Heterogeneous bioreactors 228
  Hollow-fiber bioreactors 225, 231
  Homogeneous bioreactors 225
  Microcarrier-based systems 225, 229
  Packed-bed bioreactors 225, 230
  Roller bottles 225
  Scale of operation 221, 225, 226, 230, 252
  Small-scale culture systems 221, 223
  Stirred-tank bioreactors 225
  Types of bioreactors 224, 225
  Wave bioreactors 225, 226
Biosafety 330, 331, 333, 335, 341, 342, 354
Blood coagulation 140
Blood coagulation factors 394, 398
Blood serum (*see* serum)
BSE (bovine spongiform encephalopathy) 357
Buffer exchange 305
By-product synthesis 185, 186, 188, 191, 192, 195, 199, 200, 201, 203, 208, 209, 210, 215, 426, 430

Caco-2 cells 4
Campath® 3, 396, 419
Cancer 389, 391, 392, 396, 399
Capillary electrophoresis 362
Capsid 340, 341, 439, 448, 450, 454
Carbohydrate 76, 80, 83–84, 99, 115, 130, 136, 335, 340, 484
Carbon dioxide 26, 79, 101, 103–104, 111, 115, 116, 262, 485
  Dissolved, 267
Carboxylation 103, 130, 141
Carcinogenicity 363–364
Carnitine 92
Carticel® 8
Caspases 151, 160
  Activation 160
  Classification 160, 161
  Structure 161
Cation exchangers 311–312
CBER 352
CDER 352
CDR (Complementarity-determining regions) 411, 420, 422
  Grafting 420, 421
Cell banks 4, 28, 29, 125, 332–333, 334, 355, 360, 501
Cell concentration measurement 23
Cell cycle 13, 77, 88, 96, 147, 148, 149, 150
Cell disruption (*see* cell lysis)
Cell factory 7, 8, 444
Cell growth phases 21–23
  Death phase 23
  Exponential or log phase 22
  Lag phase 22
  Stationary phase 23
Cell line storage 28–29
Cell lines 54
Cell lysis 298–299
Cell maintenance 20
Cell monitoring 263, 264, 265–267
  Flow cytometry 217, 267
  Image analysis 266, 267
  Respiration 263, 264, 265
  Size distribution 266, 267, 275, 276, 278
Cell morphology 20

Cell proliferation 147
Cell propagation 222
  Adherent cells 224
  Suspension cells 223
Cell structure
  Cytosol 14–15
  Endosome 14
  Golgi complex 14, 16
  Lysosome 14, 16
  Mitochondria 14, 16, 17
  Nucleous 14, 17
  Peroxisome 14, 17
  Plasma membrane 14
  Polyribosome 14
  Rough endoplasmic reticulum 14, 15
Cell therapy 4, 7, 8, 11, 475, 478–479, 484, 486, 489
CellCube® 225, 231, 501
CellFactory® 225, 231
Centrifugation 281
  Multi-chamber centrifuges 281, 282
  Disc centrifuges 281, 282
  Tubular centrifuges 281, 282
  Centritech® centrifuges 282
Cerezyme® 394, 398
Chemostat 83, 85, 103
Chimeric antibodies 401, 419, 420
Chimeric viral particles 454
CHO cells 2, 3, 4, 6, 10, 20, 30, 54, 55, 83, 89, 93, 95, 99–102, 125, 129, 133–134, 138, 141, 320, 322, 358, 427, 428, 429, 443
Cholesterol 91, 118, 120
Chromatin 152, 155, 156, 157, 158
Chromosomal integration 41, 53
  Positioning effect 41, 44, 53, 57
  Random recombination 44, 53
  Site-specific integration 57
Chromosome 17, 76, 493, 495
CHSE cells 81
Citrate 79, 81–82
Cleaning validation 359
Clinical trials 330, 344, 350, 352, 363–365
Clusters of differentiation (CDs) 480–483
CMP-NANA 99–100
Collagen 27, 117, 118, 119, 125, 142, 484
Collagenase 17, 21
Computational fluid dynamics 288
Continuous cell lines 443
Control (process control) 259–270
  Actuator 260, 269, 270
  Adaptive 270
  Cascade 262, 269
  Loop 260, 269
  On-off 261, 269
  PID 260, 269, 270,
  Strategies 268–270
Copper 139
Core-like particles 444
Cornea 486
CPMP 352
Cryopreservation 28, 355, 486
Cryoprotector 28
CultiSpher® 230, 444
Culture conditions 152, 153, 154, 155
Culture media
  Contaminants 29
  Degradation by light 29
Culture media types
  Basal media 111
  Chemically defined media 111, 124
  Classical formulations 112
  For insect cells 112, 463
  For mammalian cells 112
  Growth media 111
  Maintenance media 111
  Protein-free media 2, 4, 123
  Serum-free media 2, 4, 32, 111, 112, 117, 122–125, 300, 464–465
Cut size 278
  Reduced cut size 278
Cystathionine pathway 90
Cystine 87–90, 113
Cytochrome C 155, 156, 159, 166, 167, 168, 171
Cytodex™ 229, 230, 444
Cytokines 94, 352, 390, 392, 394, 400, 406, 484–485
Cytomegalovirus 357
Cytosol 14, 15, 77, 80–84, 87, 92, 94–95, 100, 496

DACS® SC 8
DAPI 157
Data treatment 186, 190–191, 192
  Experimental error 190, 213
  Rate estimation 180–191
  Smoothing methods 190–191
Deamidation 138–140
Deamination 85, 139
Density gradient 304
Deoxyglucosone 139
Desalting 305, 309
Diabodies (*see* Antibody fragments)
Diafiltration 305, 306
Dialysis 301, 305
Dipeptides 86–87
DISC (death-inducing signaling complex) 170
Disulfide bonds 130, 132, 351, 362
DMSO 28
DNA fingerprinting 356
DNA vaccines 9, 451–452

Dolichol 130, 132, 137
Doubling time 22
Downstream processing 295–324, 445, 451
Dye exclusion 23

E. coli 49–51, 62, 134–135, 494
ECGF 118
Economic aspects 400
EDTA 17, 21, 299
Efficacy of products 330, 331, 340, 342
EGF 118, 119, 120, 125
Eicosanoid 91
Elastin 484
Electrode (*see* Sensor)
Electrofocusing (*see* isoelectric focusing)
Electron microscopy 358, 496
Electrophoresis 156
Electrophoresis 310, 337–339, 341
Embryonated chicken eggs 357
Embryonic germ cells 478
Embryonic stem cells 7, 8
EMEA 350, 352, 366–367
Enbrel® 396, 406
Endoplasmic reticulum 14, 15, 130–131
Endothelial cells 4, 119, 120, 125, 479–481, 485
Endotoxin 115, 120, 360, 362
Env gene 493
Epogen® 3, 394, 402
Epstein-barr virus 5, 357, 375
Erbitux® 419
Erythropoietin 3, 6, 83, 366–367, 384, 392, 394, 400, 402, 406
Ethidium bromide 157, 158
Expanded bed adsorption 319–321
Expression systems 44–48, 55, 61, 66, 404
Extracellular matrix 27, 118, 119, 125
Exubera® 403

Fabrazyme® 394, 398
FACS (*see* Flow cytometry)
Factor IX 3, 394, 399, 402
Factor VII/VIIa 394, 399
Factor VIII 3, 139, 394, 398, 402
Factor X 129, 140–141, 399
FAD 78–80, 92
Fatty acid 79, 80, 91–95, 114
FDA 122, 351–352, 355–363, 366–367, 501
Fed-batch cultures 83, 85–86, 90–91, 138, 237
FGF 118, 119, 125
FIA (*see* Flow Injection Analysis)
Fibra-Cel™ 501
Fibroblast 4, 10, 19, 20, 24, 30, 31, 119, 120, 125
Fibronectin 21, 27, 118, 119, 125, 484
Filtration 285
  Dynamic filters 287
  Filter medium 285, 287
  Spin-filters 288
  Tangential flow filtration 285
  Rotating disc filters 288
Finite cells 4
Flavivirus 500
Flow cytometry 263, 267
Flow injection analysis (FIA) 267
Flow ratio 276
Fluorochrome 479
Follicle-stimulating hormone
Fructosamine 139–140
Fructose 77–78, 83, 116, 140
Fucosylation 133–135
Fungi 132, 136

Gag gene 493
Galactose 83, 101, 116, 131, 137
Galactosylation 99, 138
Galactosyltransferase 136
GalNac 100, 133–134, 136
Gamma-carboxylation 130, 140–141
G-CSF 366, 392, 394, 400, 402, 403
GDP 79, 89, 130
Gel filtration (*see* molecular exclusion chromatography)
Gel permeation chromatography (*see* molecular exclusion chromatography)
Gendicine® 498, 501
Gene expression 40
Gene gun 494–495, 500
Gene therapy 4, 9, 11, 31, 435, 489–493, 497–502
Genetic elements 40
  3′ untranslated region 42, 43
  5′ untranslated region 42, 43
  Capping 40, 41
  Codon usage 43
  Chromosomal elements 40
  Enhancer 40, 41, 43, 51
  Introns 41, 42
  Polyadenylation signals 41, 42, 48, 51
  Promoter 40, 41, 50, 51, 52
  Termination codon 43
  Transcription-termination sequences 41, 42, 51
Genetic engineering 374, 375, 376
GlcNac 99–100, 130–138
Glucose 24, 76–106, 113, 115–116, 118, 122, 123, 131, 137–140
Glucose transporters 77, 107
Glucosidase 130–131
Glutamate 79, 84–90, 94, 107, 114
Glutamate dehydrogenase 79, 84, 89, 101

Glutamine 76, 81–84, 91, 94–105, 113, 114, 116, 123, 137–138
Glutamine thermal degradation 116
Glutaminolysis 76, 84, 94, 99, 103
Glutathione 102
Glycation 139–140
Glycerol 28
Glycine 85, 87–89, 101, 112, 140
Glycocalyx 22, 484
Glycolic acid 496
Glycolysis 22, 35, 76–77, 79–83, 85, 89, 94–95, 97–99, 102–103, 116
Glycoproteins 405
Glycosaminoglycans 484
Glycosylation 31, 83, 97, 99–100, 103, 116, 130–138, 142, 351, 339, 340, 390, 404, 406, 448, 450
Glycosyltransferase 134–137
GM-CSF 392, 400, 402, 404
GMOs 354
GMP 349–355, 358–359
Golgi 99, 129, 131–134, 136–137, 142, 328
Gonal-f® 3, 393, 394
Granulocyte-colony stimulating factor 366, 392, 394, 400, 402, 403
Gravity settling 280
  Lamella settlers 280
  Vertical settlers 280
Growth factors 118–122, 124, 125, 149, 152, 153, 392
Growth hormone 138, 349, 366–367
Guinea pigs 357

HACA (*see* Human anti-chimeric antibody)
HAMA (*see* Human anti-murine antibody)
Hayflick limit 19, 150
HBsAg 303, 304, 448, 450
HBV 435, 441, 448, 450, 453, 454
Heart 91, 95, 475–476, 501
HEK-293 cells 4, 20, 30, 31, 141, 501
HeLa cells 20, 139, 443
Hematopoiesis 479, 484
Hematopoietic
  Cascade 483
  Growth factors 392
  Stem cells 477, 479, 481, 483–485
  Tissue 474
Hemofilia 394, 398, 399
Hemolymph 116, 118, 124, 465, 466
HEPA filters 353–354
Hepatitis 366–367, 450
Hepatitis B vaccine 3, 448, 449, 450
Hepatitis virus 357, 440
Hepatocyte 84, 96
HEPG2 cells 20
Herceptin® 3, 396, 419
Herpes virus 357, 442, 492, 500
Heterologous gene expression 44
  Baculovirus 48
  DNA virus 45
  Expression vectors 44
  Inducible promoters 50
  Multiple gene expression 53
  Plasmid vectors 50
  Quantitation of recombinant proteins 66
  RNA virus 47
  Signal peptide 52
  Stable expression 55, 56, 57
  Tags 52
  Transient expression 54, 55, 56, 57
  Viral vectors 44
Hexanoic acid 93
Hexokinase 77–78, 81, 83, 100, 110
Hexose 83
High-Five™ 32, 124, 449
Histidine 79, 88–89, 113
HIV 133, 358, 360, 452
HL-60 cell 20, 86
Hollow fiber membrane modules 286, 287
Hormones 393, 394
Human anti-chimeric antibody (HACA) 420
Human anti-murine antibody (HAMA) 418
Human genome 489, 502
Human neurotropic virus 493
Human parvovirus B19 449
Humanized antibodies 401, 419, 420
Humatrope® 402
Humira® 419
Humulin® 402
Hybridization 375
Hybridoma 2, 3, 5, 14, 30, 31, 83–86, 89–91, 93–94, 96–97, 102, 104, 119, 120, 125, 319, 375, 380, 381, 383, 410, 414, 415–418
  Hybridoma technology 410, 415–418, 420
Hydrocyclones 274, 283
Hydrodynamic stress 154
Hydrolysates 116, 123, 124
Hydrophobic interaction chromatography 301, 313–314
Hydroxyapatite 486
Hydroxylation 142
Hydroxyproline 142
Hyperglycemia 139
Hypoxia 102, 142
Hypoxia-inducible factor (HIF) 142

ICH 350, 352–353, 355
IGF-1 118, 119, 120
IGF-2 119, 120
IL-mu6 99

Immortal cell lines 4, 150
Immortalization 4, 150, 443
Immunoadhesin 99
Immunoelectrophoresis 362
Immunogenicity 403, 404, 406
Immunoglobulin 138, 411, 413, 416, 422
In vitro assays
  Comparison to *in vivo* cell growth 1, 10, 11
  Cytotoxicity assays 10, 33–36
In vivo diagnosis 394, 396, 399
InductOs™ 393, 394
Industrial cell lines 30–31
Inoculum propagation 221
Insect cells 4, 6, 24, 25, 26, 31–33, 89, 91, 103–104, 112, 116, 118, 120, 122, 124, 135–136, 262, 422, 445, 448, 449, 455 459, 461, 462, 463, 464, 465, 466, 467, 469, 470
Insulin 118, 120, 121, 124, 125
Insulin 88, 119, 120, 121, 124, 125, 375, 383, 349, 366–367
Integrity of product 331
Intellectual property 373–388
Interference RNA 497–498
Interferons 2, 3, 6, 366–368, 375, 378, 379, 384, 390, 394, 402
Interleukin 99, 118, 119, 120, 485
Introduction of exogenous DNA 58
  Calcium phosphate co-precipitation 58
  DEAE-dextran 59
  Electroporation 60
  Lipofection 60
  PEI 59
Intron A® 402
Ion exchange chromatography 301, 310–313, 314, 319, 321, 323
  Anion exchangers 311–312
  Cation exchangers 311–312
Isoaspartate 138–139
Isoelectric focusing 301, 310 338, 339, 341, 362
Isoleucine 79, 87–89
Isozyme profile 356

Karyotype 356
Keratinocytes 8, 486
Kidney 81, 96, 476
Kinetic analysis 182, 184, 185–200
Kogenate® 3, 394, 398, 399

L mouse fibroblasts 89
L929 cells 20
Labeling 404
Lactate 76, 78–87, 94–97, 101–103, 114, 116, 123, 184, 186, 188, 189, 201, 214, 496
Lactate dehydrogenase 78, 81–82, 188, 357
Laminin 27, 118, 125, 484
Langerhans islets 475
Lauric acid 93
LDH (*see* lactate dehydrogenase)
Lentivirus 9, 358, 492–493, 500
Lepidopteran 135–136
Leucine 79, 87–89, 113, 117
Leukemia 3, 500
Leukemia inhibitor factor (LIF) 485
Linoleic acid 91, 93–94, 113, 121
Linolenic acid 91, 93
Lipids 14, 15, 17, 75–76, 81–82, 91–93, 114, 116, 117, 118, 120, 125, 130, 436, 495
Lipofection 60, 500
Liposomes 9, 494–495
Liquid-liquid extraction 301, 303–304
Listeria noncytogenes 500
LLC-PK cells (porcine kidney cell) 81
Luveris® 393, 394
Lymphoblastoid cell 20, 33, 90, 102
Lymphocyte 5, 31, 94, 119, 120, 375, 454, 482, 485
Lymphocyte choriomeningitis virus 357
Lysine 87–89, 113, 139, 142, 322

Macrophages 482, 497
Maillard reaction 139
Maintenance coefficient 196, 204
Malate 79, 81–83, 101
Malate-aspartate shuttle 80–81, 95
Malic enzyme 81
Malignant transformation 19
Maltose 83, 116
Mannose 100, 116, 130–133, 135–138
Mannosidase 130–131, 137
Margaric acid 93
Mass balance 182
Master cell bank (MCB) 355–358
Mathematical model formulation 192–209
  Models for animal cell cultivation 199–209
Mathematical model types 183–185
  Non-Segregated 183, 185, 214–215
  Non-structured 183, 192–214, 215–218
  Population balance model 183, 215–218
  Segregated 215–218
  Structured 183, 185, 214–215
Mathematical model validation 213–214
MCB (*see* master cell bank)
McCoy cells 86
MDBK cells 443
MDCK cells 4, 86–87, 90, 443
Membrane adsorption 321–322
Membrane fluidity 93
Mesenchymal cells 479–481, 486
Metabolite monitoring 190, 267
Methionine 87–90, 113, 116

Methyl glyoxal 139
Methylation 134
MGH-U1 (human bladder cancer) cells 102
Microcarriers 27, 96, 229, 230, 443–446
  Macroporous 229, 230
  Manufacturers 230
  Materials 230
  Microporous 229, 230
Microfiltration 301, 305
Micro-injection 375
Mitochondria 14, 15, 16, 23, 77, 80–82, 84, 89, 91–92, 95–99, 155, 159, 162, 167, 169, 171
Mitosis 17, 75, 147–149, 477
Modes of operation 234
  Batch cultivation 235
  Continuous cultivation 240
  Continuous cultivation with cell retention (perfusion) 242
  Fed-batch cultivation 237
Molecular cloning 39
Molecular exclusion chromatography 301, 307–309, 323
Monitoring, process monitoring 184, 217, 259, 270
  In situ 260
  Off line 259, 263, 266, 267
  Online 217, 260, 265, 266, 267
  Real-time 259–260
Monoclonal antibodies 93, 139, 349, 355, 409–433, 479, 487
  Chimeric 419, 420, 421, 423
  Human 419, 421–425
  Humanized 410, 419, 420–421
  Recombinant 418–425
  Transgenic 421, 422
Monoclonal antibody production 425–430
  In vivo 410, 425, 426
  Transgenic animals 420
MRC-5 cells 4, 5, 20, 30, 31
Multiple sclerosis 391, 394
Multiplicity of infection 447, 451
Mutagenesis 492–493
Mutagenic activity 363–364
Mycobacterium leprae 498
Mycoplasma 29, 31, 121, 355–356, 360
Myeloma cell 3, 5, 20, 31, 83, 89–92, 103, 125, 358, 375, 409, 410, 414, 415, 416, 427
  Human myeloma 410
Mylotarg® 419

N-acetylglucosamine 100–101, 131
N-acetylglucosaminidase 136
N-acetylglucosamintransferase 137
N-acetylneuraminic acid 99–100
NAD 76, 79
Namalwa cells 2, 20, 124
Necrosis 23, 27, 151, colour plate/Fig. 7.3
Neulasta® 403
Neupogen® 402
Neural networks 270
Newcaste disease virus 500
NGF 119, 125
NIH 3T3 cells 20, 125
Non-viral vectors 9
  Liposomes 9
  Plasmid DNA 9, 50
Norwalk calcivirus 448
Norwalk virus 449
Notch 486
Novolin® 402
NovoSeven® 394, 399
NS0 cells 4, 20, 30, 31, 55, 427
Nucleases 494
Nucleotide sugars 130
Nutropin® 402

Octanoic acid 93
OK cells (opossum kidney cell) 81
OKT3® 418, 419, 426
Oleic acid 91, 93–94
Omalizumab 3
Omnitrope™ 367
Optimization 181, 182, 217, 426
Organ culture 34
Ornithine 85, 88–90
Ornithine transcarbamylase 500
Orthoclone® 419
Osigraft® 393, 394
Osmolality 24, 25–26, 29, 33, 111, 117, 123, 155
Osmolarity 96
Osteoblast 480, 484, 486
Ovitrelle® 393, 394
Oxidative phosphorylation 79–80, 92
Oxoaldehydes 139
Oxygen 23, 24, 26, 27, 33, 77, 80, 101–103, 111, 116, 121, 123, 139, 141–142, 189–190, 246, 261, 263–265, 428, 442
  Monitoring 259, 263–264
  Control 264, 265, 269
Oxygen supply 26–27
  Bubble free 26, 261, 263, 265
  Gas bubbling 26, 27
  Membrane diffusion 26, 263, 265
  Pressure increases 26
  Surface aeration 26, 27

Palmitic acid 93
Pancreas 476, 486
Panorex® 419
Papillomavirus 435, 448, 449

Parameter fitting 182, 184, 185, 190, 192, 193, 201, 202, 204, 206, 208, 209–213
Parvovirus 441, 448, 449, 450, 453
Patentable materials 382–384
Patents 373, 374, 375, 402, 403
  Grace period 378
  Requirements for 376–382
  Sufficiency of disclosure 381
PBM (*see* Mathematical model types)
pCO_2 (*see* Carbon dioxide, dissolved)
PDGF 118, 119, 120, 125
Peg-Intron™ 403
Pegylation 401, 403
Pentose phosphate pathway 77, 102–103
Peptide mapping 337, 340, 341
Per.C6 cells 4, 30, 31, 85
Perfusion cultivation 242
Permissible cells 436–437
pH 20, 24–26, 82, 85, 95–99, 101–104, 123, 139, 262–263, 362, 485, 496
  Buffers 24, 25
  Carbon dioxide 24, 25
  Control 24, 262
  Sodium bicarbonate 24, 25
Pharmacokinetics 365, 406
Phenylalanine 79, 88–89, 113
Phosphofructokinase 77–78, 81, 97, 100
Phospholipids 91, 93–94, 114
Phosphorylation 448
Plant cells 76, 137
Plastic bags 226–227, 444
Plate-and-frame membrane modules 286, 287
Pluronic® F68 94, 27, 120, 249
Pol gene 493
Poliovirus 443, 454, 500
Polyethylene glycol 303, 302, 314
Polyethyleneimine (PEI) 496
Polyomavirus 357, 449
Polystyrene 443, 445
Porcine parvovirus 449, 450, 453
Poxvirus 441, 500
Precipitation 300, 301, 302, 303, 304, 313, 314, 323
Primary cells 2, 4, 5, 10, 450
Primary cultures 4, 5, 17, 18, 19, 20, 119
Prions 121, 142, 357
Probe (*see* Sensor)
Process validation 129, 359
Product contaminants 329, 330, 331, 333, 335–336, 338, 340–341, 426
Product specification 335–336
Productivity 252, 253, 255
Proline 79, 85, 88–90, 101, 113, 130, 133, 139–142
Prolyl-4-hydroxylase 142
Prophylaxis 489
Propidium iodide 157, 158
Prostaglandin 91, 93
Protease 21, 298, 300, 438
Protease inhibitors 119, 300
Protein C 140–141
Protein content 337
Protein extraction 298–300
Protein hydrolysates (*see* hydrolysates)
Protein precipitation 301–303
  Precipitating agents 302
  Salting-out 302
Protein purity ranges 297
Proteoglycans 21, 484, 496
Proteolysis 297, 300, 302, 362
Protropin® 402
Puregon® 393, 394
Purification 295–324, 329, 330, 331, 335–336, 339, 340, 418, 423, 427, 445, 451
Purity 295–324, 330, 335, 336, 337, 338, 339, 342
Pyruvate 77–78, 80–83, 87, 91, 93–95, 103, 113, 115, 116, 118
  Carboxylase 81, 83
  Dehydrogenase 80–81
  Kinase 77, 81
  Phosphoenol carboxykinase 81

Quality assurance (QA) 350–351, 358–359
Quality control (QC)
  Materials 331–333
  Process 334–335
  Product 335–342

Raptiva® 419
Rebif® 391, 394
Recombinant vaccines 436, 453
Regulatory agencies 389, 404
Regulatory aspects 329, 331, 349–372
Remicade® 396, 401, 419
ReoPro® 419
Reporter markers 64
  Alkaline phosphatase (AP) 65
  Beta-galactosidase (β-gal) 64
  Chloramphenicol acetyl transferase (CAT) 64
  Green fluorescent protein (GFP) 65
  Luciferase 65
Residual cellular DNA 361
Retractable housing 263
Retrovirus 5, 9, 31, 119, 358, 439, 440, 442, 492–493, 500
Reverse phase chromatography 313–314
Reverse transcriptase 358, 439, 493
Ribosome 15, 16, 17, 130, 133, 438
Rituxan® 419

Roller bottle 444
Rotavirus 449, 454

S2 cells 32
Saccharomyces cerevisiae 83, 136, 500
Salmonella typhimurium 500
Salts 112, 113, 114, 117, 122, 124
Scale-up 250, 444
Schiff's base 139
SCID mice- 422
SCID syndrome 9, 500
SDS-PAGE 301, 310, 338, 341, 362
Selection markers 61
  Biochemical markers 61
  Gene amplification 61
  Morphological changes 61
Semi-adherent cells 21
Semliki forest virus 500
Senescence 150
Sensors, principle of 263, 264, 266, 267, 417
Separation efficiency 274
  Total separation efficiency 275, 276
  Reduced total efficiency 276
  Grade efficiency 277
  Reduced grade efficiency 278
Sequon 130–133
Serine 85, 87, 89–90, 113, 130, 133–134
Serum 117–121
  As a cell protecting agent 118, 121
  Components 118
  Functions 117
  Risks associated to its use 121
  Substitutes 27
Serum albumin 92, 125
Serum-free media 2, 4, 32, 85, 87, 92–94, 102, 111, 112, 117, 122–125, 300, 358, 501
Set-point 260, 269, 270
Sf-21 cells 449–451
Sf-9 cells 32, 89, 135, 449–451
Shear sensitivity 349, 445
Shear stress 154
SH-SY5Y cells 20
Sialic acid 100–101, 131, 135, 137–138
Sialylation 98–99, 103, 133–134, 136, 138
Sialyltransferases 136–137
Simian 40 virus 500
Simulect® 3, 396, 419
Site-directed mutagenesis 406
Size exclusion chromatography (*see* molecular exclusion chromatography)
Solid support (*see* substrata for cell adhesion)
Sp2/0 cells 4, 55, 83, 416, 417, 427
SP2Ag14/0 cells 416, 417, 427
Specific rate 182, 186–188, 261, 266
  Death 186, 187, 203, 205, 211
  Growth 183, 184, 187, 191, 193–196, 428
  Product synthesis 181, 187, 188, 198, 199, 201, 205, 207, 208
  Substrate consumption 188, 189, 190, 196–197, 204–205
Spheroids 34
Sphingolipids 91
Spodoptera frugiperda cells 32, 89, 135, 449, 451
Stability 330–333, 335, 337, 339, 423
Stearic acid 93
Stem cells 4, 7, 8, 475–487
Sterols 91
Stirring (*see* Agitation, Oxygen supply)
Substrata for cell adhesion 27
Substrate monitoring 190, 267
Succinamide 138
Sulfation 134
Superoxide dismutase 102
Suspended cells 21
SV-40 42, 45, 49, 51, 55, 449
Synagis® 419

T antigen 136
T lymphocytes 418, 422, 425
TCA cycle 78–85, 87–92, 97, 99, 101, 103, 115
Temperature 26, 28, 101, 103, 110, 138–139, 259, 261–262, 355, 362, 485
  Cell growth 26
  Control 260, 261–262, 269
  Cryopreservation 28
  Upon thawing 28
Tenecteplase™ 394, 398, 406
Teratogenic activity 364
Teratomas 8
Terminal settling velocity 274, 280, 281
Tetrahydrofolate 89
TGF 119
Therapeutic enzymes 393, 394
Therapeutic proteins 389–408
Thermal degradation 86
Threonine 88–89, 101, 113, 133–134
Thrombospondin 484
Time of infection 447
Time-to-market 390, 402
Tissue engineering 8
Tissue plasminogen activator 2, 101, 133, 322
Toxicity 363–364, 492
Transamination 94
Transdifferentiation 475
Transesterification 92
Transformed cells 19
Trastuzumab 3, 6
Trimera mouse 422
Trypsin 17, 21, 356–357
  inhibitor 118

trypsinization 22, 32, 33
Tryptophan 29, 88–89, 113
Tumor necrosis factor 99
Tumor-suppressing gene (p53) 501
TUNEL 156

U 266B1 cells 20
U937 cells 20
UDP-GlcNAc 100, 130, 138
UDP-GNAc 97, 99
Ultrafiltration 301, 305–306
Ultrasonic separators 290
Umbilical cord 485–486
Urea 96

Vaccinia virus 500
Valine 88–89, 113
VEGF 485
Vectors (*see* Heterologous gene expression, Non-viral vectors, Viral vectors)
Vero cells 2, 4, 20, 86, 443, 444, 445, 446, 447
Viral antigens 435
Viral vaccines 2, 435, 454
Viral vectors 9, 44–50, 435, 491–492, 498, 500–502
Virus 2, 4, 44–50, 121, 436–442, 140, 355–361, 371, 491–493, 500
Adsorption to cells 437
Genome structure 437–441
Infection 442
Internalization 437
Replication 436–437
Structure 436, 447
Virus-like particles (VLPs) 2, 7, 447, 453–455
Vitamin K 117, 141
Vitamins 113, 114, 117, 120, 122, 123, 125

Water quality 114–115
WCB (*see* Working cell bank)
WEHI 231 cells 20
Western blotting 362
WHO 350, 353–362
WI-38 cells 2, 4, 5, 102
Working cell bank (WCB) 355–357

Xenotransplants 475–476
Xigris® 394, 402
Xolair® 3, 396, 419
Xylose 135, 137

YAC 1 cells 20
Yield coefficient 186, 188–189

Zenapax® 396, 400, 419
Zevalin® 419