

HORMONAL PROTEINS AND PEPTIDES

Edited by CHOH HAO LI

*The Hormone Research Laboratory
University of California
San Francisco, California*

VOLUME II



ACADEMIC PRESS New York and London 1973

A Subsidiary of Harcourt Brace Jovanovich, Publishers

CONTRIBUTORS

MIKLOS BODANSZKY
JOHANNES MEIENHOFER
J. RAMACHANDRAN

COPYRIGHT © 1973, BY ACADEMIC PRESS, INC.

ALL RIGHTS RESERVED.

NO PART OF THIS PUBLICATION MAY BE REPRODUCED OR
TRANSMITTED IN ANY FORM OR BY ANY MEANS, ELECTRONIC
OR MECHANICAL, INCLUDING PHOTOCOPY, RECORDING, OR ANY
INFORMATION STORAGE AND RETRIEVAL SYSTEM, WITHOUT
PERMISSION IN WRITING FROM THE PUBLISHER.

ACADEMIC PRESS, INC.

111 Fifth Avenue, New York, New York 10003

United Kingdom Edition published by

ACADEMIC PRESS, INC. (LONDON) LTD.

24/28 Oval Road, London NW1

LIBRARY OF CONGRESS CATALOG CARD NUMBER: 72-88367

PRINTED IN THE UNITED STATES OF AMERICA

List of Contributors

Numbers in parentheses indicate the pages on which the authors' contributions begin.

MIKLOS BODANSZKY (29), Department of Chemistry, Case Western Reserve University, Cleveland, Ohio

JOHANNES MEIENHOFER (45), The Children's Cancer Research Foundation, and Department of Biological Chemistry, Harvard Medical School, Boston, Massachusetts

J. RAMACHANDRAN (1), The Hormone Research Laboratory, University of California, San Francisco, California

Peptide Synthesis: A Review of the Solid-Phase Method

JOHANNES MEIENHOFER

I.	Introduction	48
II.	The Solid-Phase Concept	54
	A. Basic Features	54
	B. Experimental Scheme	56
	C. Intrinsic Problems	58
	D. Related Methods	66
III.	The Chemistry	72
	A. The Solid Support and Introduction of Functional Groups	72
	B. Attachment of Amino Acids or Peptides	82
	C. Protection-Deprotection	95
	D. Coupling Methods	114
	E. Removal of the Solid Support	135
	F. Purification and Characterization	146
IV.	The Operation	155
	A. Reaction Vessel and Stirring	157
	B. Solvents and Reagents	163
	C. Schedule for Synthesis	164
	D. Analytic Control	169
	E. Mechanization and Programming	175
V.	Applications	178
	A. Model Peptides	179
	B. Peptides with Biological Functions	179
	C. Special Applications of the Solid-Phase Method	191
	D. Other Polymeric Products	221
VI.	Comparison and Choice between Solid-Phase and Solution Synthesis	221
	A. Present State of Solid-Phase Synthesis	221
	B. Present State of Solution Synthesis	227
	C. Project-Oriented Choice of Method	239
	D. Outlook	243
	References	244

Abbreviations

The abbreviations used for amino acids and protecting groups and the designation of peptides and side-chain linkages follow the rules of the IUPAC-IUB Commission on Biochemical Nomenclature, in *Biochemistry* 5, 1445 and 2485 (1966). The rules for naming synthetic modifications of natural peptides have been described in *Biochemistry* 6, 362 (1967). The following additional abbreviations have been used:

A. AMINO AND HYDROXY ACIDS

Aad	L-2-aminoadipic acid	Gly*	[¹⁴ C]glycine
Abu	L- α -aminobutyric acid	Har	<i>homo</i> -L-arginine
Acp	ϵ -aminocaproic acid	Ile*	[¹⁴ C]isoleucine
Aev	L- α -amino- β -ethylvaleric acid	Lac	L-lactic acid
Aib	α -aminoisobutyric acid	Leu*	[¹⁴ C]leucine
Apb	3-amino-4-phenylbutyric acid	MePro	<i>trans</i> -3-methylproline
Apib	3-amino-3'-phenylisobutyric acid	Met(O)	methionine sulfoxide
Aud	ω -aminoundecanoic acid	Nle	L-norleucine
Avl	δ -aminovaleric acid	Pab	<i>p</i> -aminobenzoic acid
Cha	L-cyclohexylalanine	Phg	D-C-phenylglycine
Chg	L- α -cyclohexylglycine	Pic	L-pipecolic acid
Cle	cycloleucine, 1-aminocyclopentanecarboxylic acid	Plc	phenyllactic acid
Cpg	L- α -cyclopentylglycine	Pro*	[¹⁴ C]proline
Cys(CM)	S-carboxymethylcysteine	<Glu	pyroglutamic acid, L-pyrrolidone-2-carboxylic acid
FPro	<i>trans</i> -4-fluoroproline	Thi	β -2-thienyl-D, L-alanine
		Tyr(Me)	O-methyltyrosine
		d-Val	deuterated L-valine

B. PROTECTING AND ACYLATING GROUPS

Ac	acetyl	Dnboc	2,2'-dinitrodiphenylmethyl-oxycarbonyl
Acm	acetamidomethyl	Dnp	2,4-dinitrophenyl
Adoc	adamantyloxycarbonyl	Dns	dansyl, 5-dimethylamino-naphthalene-1-sulfonyl
Alk	alkyl	Dpm	diphenylmethyl
Aoc	<i>tert</i> -amyloxycarbonyl	Ec	ethylcarbamoyl
Bmv	2-benzoylmethylvinyl	Et	ethyl
Boc	<i>tert</i> -butyloxycarbonyl	Fmoc	9-fluorenylmethoxycarbonyl
Bpoc	2-(<i>p</i> -biphenyl)isopropyl-oxycarbonyl	Foc	furfuryloxycarbonyl
Btm	benzylthiomethyl	For	formyl
Bu ^t	<i>tert</i> -butyl	Mbh	4,4'-dimethoxybenzhydryl
Bz	benzoyl	Meb	<i>p</i> -methoxybenzyl
Bzl	benzyl	Meoz	<i>p</i> -methoxybenzyloxycarbonyl
Dim	dimedonyl, 5,5-dimethyl-cyclohex-2-en-1-one-3-yl	Mpr	β -mercaptopropionyl
Dimoz	3,5-dimethoxybenzyloxycarbonyl	Mud	β -mercaptopundecanoyl
		Mvl	δ -mercaptopaleryl

NO ₂ •Z	<i>p</i> -nitrobenzyloxycarbonyl	OPic	4-picolyl ester
Nps	<i>o</i> -nitrophenylsulfenyl	OSu	<i>N</i> -hydroxysuccinimide ester
OBu ^t	<i>tert</i> -butyl ester	Pbu	phenylbutyryl
OBzl	benzyl ester	Pel	pelargonyl
Oct	octanoyl	Pth	phthalyl
OEt	ethyl ester	Prot	protecting group
OMe	methyl ester	Tfa	trifluoroacetyl
OMtp	methylthiophenyl ester	Thp	tetrahydropyranyl
ONb	<i>p</i> -nitrobenzyl ester	Tos	<i>p</i> -toluenesulfonyl
ONm	<i>m</i> -nitrophenyl ester	Tri	triphenylmethyl
ONo	<i>o</i> -nitrophenyl ester	Z	benzyloxycarbonyl
ONp	<i>p</i> -nitrophenyl ester	Ztf	2,2,2-trifluorobenzyloxy-carbonylaminoethyl
OPcp	pentachlorophenyl ester		

C. OTHER ABBREVIATIONS

ACTH	adrenocorticotropin	MeOH	methanol
Ar	aryl	MSH	melanotropin
CHA	cyclohexylamine	NEPIS	<i>N</i> -ethyl-5-phenylisoxazolium-3'-sulfonate
CDI	<i>N,N'</i> -carbonyldiimidazole	NEt ₃	triethylamine
DCCI	dicyclohexylcarbodiimide	Resin*	copolystyrene-2% divinylbenzene*
DCHA	dicyclohexylamine	RNase	ribonuclease
DEAE	diethylaminoethyl	SDS	sodium dodecyl sulfate
DMF	dimethylformamide	SE	sulfoethyl
DVB	1,4-divinylbenzene	SOL	solution synthesis
EDC	1-ethyl-(3-dimethylaminopropyl) carbodiimide x HCl	SPS	solid-phase synthesis
EEDQ	<i>N</i> -ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline	TEA	triethylamine
EtOH	ethanol	TFA	trifluoroacetic acid
HOAc	acetic acid (AcOH)	TMV	tobacco mosaic virus
HOSu	<i>N</i> -hydroxysuccinimide	TRF,	
MA	mixed anhydride	TRH	thyrotropin releasing factor

Arrangement of Peptides in the Tables

The model peptides are listed by size. Within each group the system of Goodman and Kenner (1957) is followed, i.e., alphabetical order of amino acid residues starting from the C-terminal end.

Biologically active and related peptides have been grouped under (a) hormones, (b) kinins, and (c) others. Within these groups the order is alphabetical by (trivial) name. Within each name the order is generally: (1) naturally occurring peptide(s), (2) analogs, homologs, derivatives, and (3)

* The terminology which has become established for derivatives of copolystyrene-divinyl benzene and for various linkages with amino acids or peptides is used. It is not always consistent depending on whether the benzene ring is included in a term or not.

partial sequences. Analogs and derivatives have been ordered by degree of substitution, over the (first) position number, over alphabetical order by the Goodman and Kenner convention.

The tables do *not* contain a *complete* listing of all peptides prepared by solid-phase synthesis. Furthermore, an effort has been made to list only the largest (final) peptide of any *one* synthesis. Literature covered until June 1971.

The common amino acids are of the L configuration unless otherwise indicated.

I. Introduction

Several recent reports on chemical syntheses of proteins have attracted wide interest and publicity as milestones of biochemical research. As a result, increased attention has been paid to peptide synthesis, a specialized field of organic chemistry. At the same time the definitive structure identification of some very small isolated peptides, such as the thyrotropin releasing hormone (TRH) or the melanotropin-release inhibiting factor, required peptide synthesis to come to the aid of analytic investigations. These and other comparatively small peptides possess extremely high biological potency as hormonal control factors. The biological roles and the chemical structures of an increasing number of peptide and protein hormones are being elucidated, and it appears that many other important biological functions of peptides are still to be determined, e.g., in nerve and brain function. The increased visibility of peptides has given further impetus to peptide synthesis. Several excellent books have been published on the subject which give descriptions of the historical developments, of the numerous contributions to the presently available arsenal of synthetic methods, of the delineation of strategies and tactics of peptide synthesis, of the many actual syntheses of peptides, and of their chemical and biological properties (Greenstein and Winitz, 1961; Schröder and Lübke, 1965, 1966; Bodanszky and Ondetti, 1966; Jakubke and Jeschkeit, 1969; Law, 1970). These reviews present an enormous number of synthetic procedures for what would outwardly appear to be a simple matter: the repeated formation of the same chemical bond. Experimentally, however, differences in the ease of formation of a given peptide bond can be tremendous, depending on the neighboring sequences—just as sequence characteristics confer upon peptides specific physical, chemical, and biological properties, of the known immense variety. Delightful as the legion of methods described in above reviews is to the adept, it frequently causes no small confusion or irritation to the uninitiated. On the other hand, synthetic peptides, in particular those with biological activity, have become of great interest to investigators in many branches of science

and medicine. However, for a long time well-defined synthetic peptides have not been readily available.

With the development of the solid-phase method during the past decade, this situation has changed because an easy and convenient experimental procedure for peptide synthesis became available. When Merrifield (1962, 1963) published his first accounts on the novel approach, he demonstrated its practical usefulness by the preparation of the crystalline tetrapeptide, L-leucyl-L-alanyl-glycyl-L-valine. The potential of the procedure for rapid synthesis of biologically active peptides was shown a year later by preparations of 100–500 mg amounts of bradykinin (Merrifield, 1964a,b). Solid-phase peptide synthesis offered many advantages over conventional solution synthesis, in particular, simplicity of operation and dramatic savings in time. The method appeared to provide the ideal means for peptide and protein synthesis. It attracted quickly wide attention and has found numerous applications not only by peptide chemists but also, and perhaps even more, by biochemists and biologists, pharmacologists, physiologists, endocrinologists,

Table I—Reviews and Reports about Solid-Phase Peptide Synthesis

Merrifield, R. B. (1965)	(a) <i>Science</i> 150 , 178–185. (b) <i>Endeavour</i> 24 , 3–7.
(1966)	In "Hypotensive Peptides" (E. G. Erdös, N. Back, and F. Sicuteri, eds.), pp. 1–13. Springer-Verlag, Berlin and New York.
(1967)	<i>Recent Progr. Horm. Res.</i> 23 , 451–482.
(1968)	(b) <i>Sci. Amer.</i> 218 , 56–73.
(1969)	(a) <i>Advan. Enzymol.</i> 32 , 221–296. (b) <i>J. Amer. Med. Ass.</i> 210 , 1247–1254.
(1970)	<i>Annu. Rev. Biochem.</i> 39 , 841–866. (With A. Marglin).
Ferriere, N. (1966)	<i>Sci. Progr., Nature</i> (Paris), No. 3372 127 (in French).
Halstrøm, J. (1967)	<i>Saettryk Dan. Kemi</i> 48 , 59–63 (in Danish).
Arnold, Z. (1968)	<i>Farm. Pol.</i> 24 , 371–376 (in Polish).
Izdebski, J., and Drabarek, S. (1968)	<i>Wiad. Chem.</i> 22 , 35–43 (in Polish).
Okuda, T. (1968)	<i>Naturwissenschaften</i> 55 , 209–211 (in German).
Vesa, V. C. (1968)	<i>Usp. Khim.</i> 37 , 246–255 (in Russian).
Jouzier, E. (1969)	<i>Prod. Probl. Pharm.</i> 24 , 313–321 (in French).
Stewart, J. M., and Young, J. D. (1969)	"Solid Phase Peptide Synthesis." Freeman, San Francisco, California.
Shimonishi, Y. (1969)	<i>Tampakushitsu, Kakusan, Koso</i> 13 , 651–658 (in Japanese).
Losse, G., and Neubert, K. (1970a)	<i>Z. Chem.</i> 10 , 48–64 (in German).
Sheppard, R. C. (1973)	In "Peptides 1971" (H. Nesvadba, ed.). North-Holland Publ., Amsterdam (in press).

and immunologists. Many reviews have been published, as compiled in Table I. A comprehensive description of concept and methodology by Merrifield (1969a) and a book describing experimental details and operational instructions (Stewart and Young, 1969) are available.

Soon solid-phase synthesis aroused vocal criticism from many peptide chemists who have generally not taken issue with the basic concept of the new method but rather with the ill-defined purity of some of the products. Unless the incorporation of an amino acid residue is quantitative, some unreacted lower peptide will be retained in the solid-phase. With the combined effects of repetition at every step and of accumulation (removal is not possible until after completion of the synthesis), complex mixtures of "micro-heterogeneous" nature are formed.

However, investigators in biological or biochemical areas of work have been enthusiastic about the method. They have used products prepared by solid-phase synthesis in a variety of applied experimental or biological studies with apparent satisfactory results.

The difference in conclusions reached from the use of the method might be rationalized from considerations of vastly differing backgrounds and objectives. Thus *peptide chemists* have prepared homogeneous peptides, traditionally through purification of intermediates rather than through intensive purification of final products. They expected too much from solid-phase synthesis too early and were disappointed when the products, particularly larger peptides, showed heterogeneity which was unacceptable by established standards. *Biologists*, on the other hand, have very likely been unaware of possible pitfalls and by-product formation during chemical synthesis of peptides. They did, indeed, not expect very much and were, consequently, delighted with their progeny. By one or another quick and insensitive test they relieved themselves of the required proofs of identity and/or purity. *Biochemists*, furthermore, have frequently acquired a solid background in fractionation of multicomponent mixtures from natural isolates. This experience has allowed them to purify some crude solid-phase synthetic peptides, at least up to decapeptides, to high degrees of purity. (In favorable cases, even pentadeca—or up to eicosapeptides appear to have been obtained in rather high degrees of purity.)

The controversy over these and other aspects of solution synthesis versus solid-phase synthesis grew at times into rather heated polemics. Two confronting camps formed, but the lines were not very rigid. Synthetic achievements have been made through both approaches. The results have helped to assess specific merits as well as limitations of each. A selection of the largest peptides which have been prepared during the past 8 years by solid-phase-synthesis and by solution synthesis is shown in Table II for comparison.

To date the largest peptide prepared by solution synthesis is ribonuclease

S-protein possessing 104 amino acid residues. Total syntheses of two adrenocorticotropins (human and bovine), of several calcitonins and insulins, and of glucagon and secretin have been achieved. Solid-phase synthesis afforded proteins prepared according to target sequences for human growth hormone, bovine ribonuclease A, an analog of horse heart cytochrome c, ribonuclease T₁, and acyl carrier protein.

It is apparent from this comparison (Table II) of selected examples that both solid-phase synthesis and solution synthesis have produced results which surpassed expectations of only a few years ago. It is also apparent that a stage of development has now been reached where it might be considered unwise judgment to rely principally and categorically on one of the two approaches alone and to dismiss the other as either useless or unmanageable.

Table II—Examples of Achievements by Solid-Phase and by Solution Peptide Synthesis

Solid-Phase Synthesis		Solution Synthesis	
Peptide and Reference	Size ^a	Peptide and Reference	Size ^a
Human growth hormone Li and Yamashiro (1970)	188 ^b	Ribonuclease S-protein Hirschmann <i>et al.</i> (1969)	104
Ribonuclease A Gutte and Merrifield (1969, 1971)	124	Adrenocorticotropin Porcine: Schwyzer and Sieber (1963)	39
Cytochrome c Sano and Kurihara (1969)	104	Human: Bajusz <i>et al.</i> (1967)	
Ribonuclease T ₁ Izumiya <i>et al.</i> (1971)	104	Ribonuclease T ₁ -(12-47) Beacham <i>et al.</i> (1971)	35
Acyl carrier protein-(1-74) Hancock <i>et al.</i> (1971) Marshall <i>et al.</i> (1973)	74	Calcitonin Porcine: Rittel <i>et al.</i> (1968) Guttman <i>et al.</i> (1968)	32
Bovine basic trypsin inhibitor Noda <i>et al.</i> (1971) Izumiya <i>et al.</i> (1972)	58	Human: Sieber <i>et al.</i> (1968) Salmon: Guttman <i>et al.</i> (1969)	
Ferredoxin Bayer <i>et al.</i> (1968a,b)	55	Insulin	30 21
Staphylococcal nuclease T-(6-47) Ontjes and Anfinsen (1969a)	42	Meienhofer <i>et al.</i> (1963) Katsoyannis <i>et al.</i> (1964) Kung <i>et al.</i> (1965)	
Parathyroid hormone-(1-34) Potts <i>et al.</i> (1971)	34	Glucagon Wünsch (1967)	29
Insulin	30 21	Secretin Bodanszky <i>et al.</i> (1966)	27
Marglin and Merrifield (1966)			

^a Amino acid residues.

^b According to the originally proposed structure (Li *et al.*, 1966; 1969). For a revised complete structure, see Li and Dixon (1971).

It would be misleading to attribute the exponential increase in synthetic achievements solely to progress in methodical developments. The decisive impetus and direction which modern peptide chemistry received from du Vigneaud's first oxytocin synthesis (du Vigneaud *et al.*, 1953) has been amplified and accelerated by a steadily increasing number of areas of applications. In addition to such classical motivations as proof of structure through synthesis and establishment of correlation between chemical structure and biological activity, it has become possible to synthesize peptides with changed specificity of action (inhibitors) and to produce peptide hormones commercially by total synthesis (oxytocin, modified ACTH fragments). Recently, peptide synthesis has been used to provide homogeneous preparations for conformational analysis (oxytocin, vasopressin, angiotensin), or to establish the structure of an active principle (thyrotropin releasing factor, TRF) occurring naturally in extremely small amounts. Furthermore, it appears as if synthesis might eventually have to provide for rapidly increasing needs of peptide therapeutics whose natural supply is presently still adequate but destined to remain limited (insulin).

A basic scheme has been followed in peptide synthesis which proceeds through three stages, as shown in Fig. 1. It begins with the preparation of protected amino acids. The most commonly used amino protecting groups are the benzyloxycarbonyl group (Bergmann and Zervas, 1932), the *tert*-butyloxycarbonyl group (Anderson and McGregor, 1957), and modifications of these groups. For carboxyl protection methyl, ethyl, benzyl, and *tert*-butyl esters are most frequently employed. In addition, side-chain protection often is required for trifunctional amino acids. The actual peptide bond formation (second stage) is accomplished by activation of the carboxyl group in the carboxyl component followed by reaction with the amine component to produce a protected peptide. Partial deprotection in the third stage affords intermediates for further condensation. Complete deprotection at the end of a synthesis provides the desired free peptide.

Four efficient methods of peptide bond formation have found wide and general application during the past decade (compare, for a brief review, Meienhofer, 1962): the azide method (Curtius, 1902); the mixed anhydride method (Wieland and Bernhard, 1951; Boissonnas, 1951; Vaughan, 1951); the dicyclohexylcarbodiimide method (Sheehan and Hess, 1955); and the active ester method (e.g., nitrophenyl esters: Bodanszky, 1955).

The choice of protecting groups, of condensing agents, and of deprotection procedures is regarded as the *tactics* of peptide synthesis, while the pattern of assembling all amino acid residues into the desired peptide sequence is referred to as the *strategy* (Bodanszky and Ondetti, 1966). The two principal strategies "fragment condensation" and "incremental (stepwise) chain

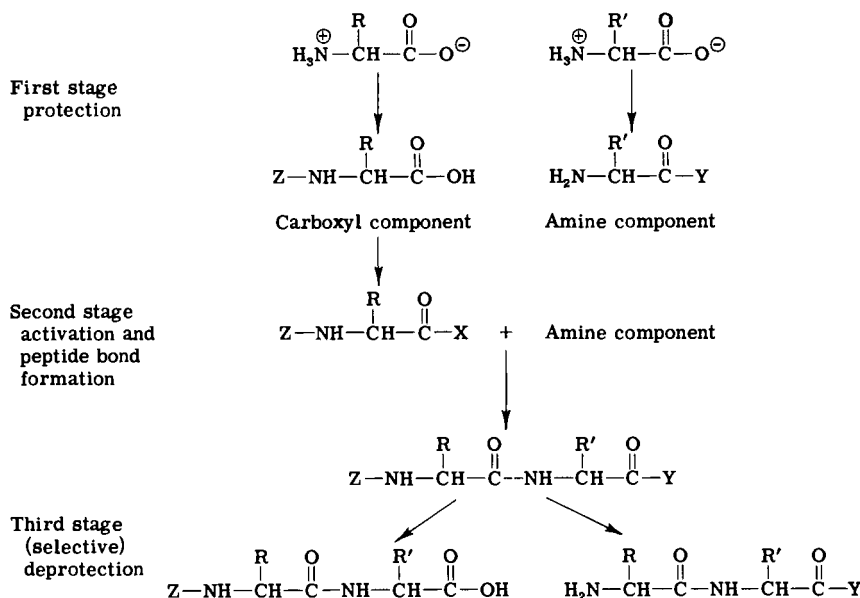


FIG. 1. General scheme of peptide synthesis.

elongation" (Bodanszky and du Vigneaud, 1959; Bodanszky *et al.*, 1960) are schematically depicted in Fig. 2. Fragment condensation allows greater flexibility in the choice of protecting groups and condensing methods but is impeded by the *danger of racemization* at the α carbon of the C-terminal amino acid in the carboxyl component.

The above fundamentals of peptide synthesis have been briefly reviewed because the solid-phase method "depends primarily on the chemistry of conventional peptide synthesis. The protecting groups and coupling reactions are those which have slowly evolved over the past 70 years, and many of the new developments in peptide chemistry will also have application to the new method. The principal change from the chemistry of classical syntheses is due to the introduction of the solid support . . ." (Merrifield, 1969a). Therefore, many of the discussions about the solid-phase method require an understanding of the basic scheme and of the strategies in Figs. 1 and 2, respectively, and of the common methodology of solution synthesis.

The above mentioned recent trends in peptide chemistry: preparation of increasingly larger molecules and the chemical synthesis of proteins, along with the great potential of applications that appear to open up with the newly discovered small peptides of high biological potency, clearly indicate that the role of peptide synthesis as an integral part of biological research in

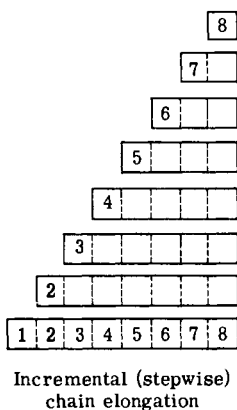
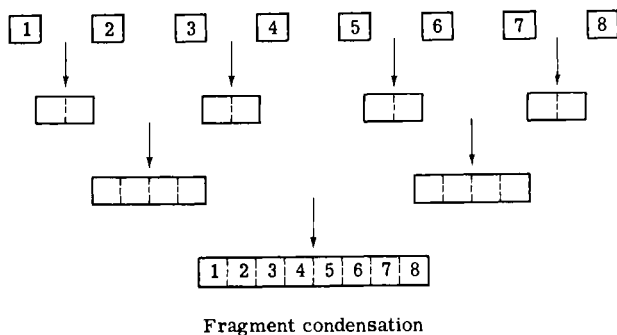


FIG. 2. Strategies of peptide chain assembly.

many areas will rapidly grow in importance. The question remains: which approach should be used with advantage? It will be attempted in this review to show that certain projects of peptide synthesis can effectively be accomplished by solid-phase synthesis, and others must be carried out by solution synthesis. Ideally, the decision should be a matter of judicious choice between available alternatives, and the choice should depend on the nature and objective of the individual study.

II. The Solid-Phase Concept

A. BASIC FEATURES

The basic features of Merrifield's procedure involve *phase separation* of the peptide from reagents, by-products, and side products. The growing

peptide chain is kept covalently attached to an entirely insoluble support throughout all stages of the synthesis. The solid phase must be in a form which allows *rapid filtration* for the removal of the liquid phase, containing the soluble reagents, by-products, and side products, from the solid phase. To be applicable for multistage syntheses of large polypeptides, *100% quantitative incorporation* of each amino acid residue must be accomplished. Finally, the solid support should be *fully removable* from the completed peptide without altering or degrading the desired product. Complete achievement of the above essentials should provide a procedure for the rapid preparation of homogeneous peptides in high yields.

The general plan adopted for the practical implementation is shown in

Strategy	Experimental operation	Solid-phase reactions	Solvating medium (solution)
Single initial stage	Functionalization	SOLID	Anchor group reagent Derivatizing agent
	Attachment	X-A-SOLID $\text{Prot-HNCHR}_1\text{CO-A-SOLID}$	Prot.-amino acid Reagent Swelling solvent
Repetitive chain elongation stage (cycle)	Deprotection	$\text{H}_2\text{NCHR}_1\text{CO-A-SOLID}$	Deprotecting agent Cleaved protection products Acid, base, salts
	Coupling	$\text{Prot-NHCHR}_2\text{CO-HNCHR}_1\text{CO-A-SOLID}$	Prot.-amino acid Coupling reagent By-products
Single final stage	Removal Purification	SOLID	Acid, base, solvent Peptide (crude) $\Downarrow$ Product

FIG. 3. A general scheme of solid-phase peptide synthesis. Abbreviations: A, anchoring chain between resin and peptide; X, functional group for the attachment of the first amino acid residue.

Fig. 3. A suitable functional group, X, is introduced into the solid support of choice through an anchoring chain, A. The first (C-terminal) protected amino acid is attached to the solid support through reaction with the functional group X. "Functionalization" of the solid material and "attachment" are the first two nonrepetitive steps. For the assembly of the peptide the strategy of incremental (stepwise) chain elongation (Fig. 2, bottom) is used, which is characterized by the incorporation of one amino acid residue at a time starting from the C-terminal end (Bodanszky and du Vigneaud, 1959). Selective cleavage of the α -protecting group ("deprotection") is followed by the introduction of the second protected amino acid ("coupling"). Alternating successive repetitions of deprotection and coupling steps, called "cycles," effect the actual peptide chain elongation. The final stage of a synthesis consists in removal of the solid support after cleavage of the covalent peptide to polymer bond and in purification of crude products.

The procedure offers three principal advantages over solution synthesis: (1) losses are avoided, which occur during isolation and purification of each intermediate through recrystallization, reprecipitation, or other fractionation procedures; (2) reactions may be driven to completion through the use of large excesses of reactants in the liquid phase; and (3) no solubility problems arise. Limited solubility or insolubility constitute the most serious experimental obstacles for solution synthesis of many peptides, especially when they contain more than twenty to thirty amino acid residues. An additional favorable feature over solution synthesis is the use of a single reaction vessel throughout all repetitive operations.

The practical advantages of the solid-phase procedure over solution synthesis are: unprecedented speed, simplicity of operation, and possibility for automation.

B. EXPERIMENTAL SCHEME

For the experimental application of the general plan, discussed above (Fig. 3), Merrifield selected protecting groups and reagents of proven usefulness in conventional peptide synthesis. The solid support of choice is beaded copolystyrene-2% divinylbenzene which can be chloromethylated. The attachment of the first amino acid through a benzyl ester bond is effected by refluxing a mixture of the chloromethylated resin and the $N\alpha$ -protected amino acid in ethanol in the presence of base (Fig. 4). For the protection of the α -amino function, the *tert*-butyloxycarbonyl group is mostly used. Deprotection is effected with hydrogen chloride in acetic acid or dioxane or with anhydrous trifluoroacetic acid (in methylene chloride) leaving the amino groups in protonated form. These are converted into free amino groups by treatment with triethylamine in methylene chloride. The

3. SOLID-PHASE PEPTIDE SYNTHESIS

57

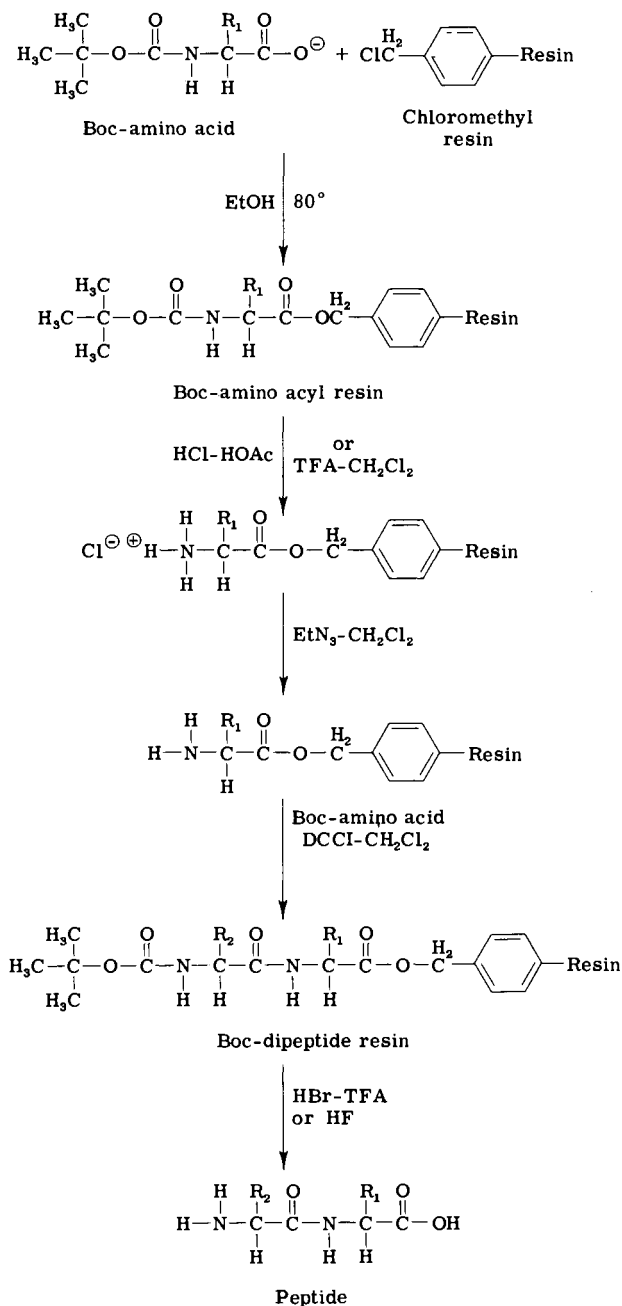


FIG. 4. Standard experimental scheme for solid-phase peptide synthesis (Merri-field, 1964b). Resin, copolystyrene-2% divinylbenzene.

deprotection step thus requires two successive operations. Coupling with the next *tert*-butyloxycarbonylamino acid is carried out in methylene chloride with the aid of dicyclohexylcarbodiimide (Sheehan and Hess, 1955) using fourfold molar excess of reactants* and 2–6 hour reaction periods. Dicyclohexylcarbodiimide is ideally suited because of its high reactivity. As a so-called condensing reagent it can be simply added to component mixtures and thus allows easy and convenient manipulation. After completing the required number of repetitive cycles, the desired peptide is removed from the resin. To achieve this, the benzyl ester bond is cleaved by treatment with hydrogen bromide in anhydrous trifluoroacetic acid (Merrifield, 1964a) or, alternatively, by anhydrous hydrogen fluoride in the presence of anisole (Lennard and Robinson, 1967).

The above described procedure is often referred to as the standard Merrifield procedure (Merrifield, 1964b,c; Marshall and Merrifield, 1965). For the great majority of reported solid-phase syntheses this or essentially similar procedures have been used. Numerous minor variations have been made to optimize the yield and homogeneity. Among the most frequently changed parameters are reaction time, solvents for reactions and washings, and repeated reactions. More substantial modifications such as the use of different protecting groups or other reagents for deprotection, coupling, or removal of the solid support will be discussed later (Sections III,C–E). The most salient features of this experimental scheme are its simplicity and its universality. A series of simple successive washing operations has replaced those parts of solution synthesis which have always placed the highest demands on the expertise and specialized experience of the investigator, i.e., (a) separation of the peptide intermediate from unreacted starting materials, reagents, by-products, undesired side products or artifacts, and (b) purification of isolated peptide intermediates after each step. A rather dramatic reduction of workload is attained. Moreover, the repetitive nature of successive operations allows mechanization through programmers and timers.

Indeed, Merrifield's motivation in developing the solid-phase method has been the belief in "a need for a rapid, quantitative, automatic method for synthesis of long-chain peptides" (Merrifield, 1969b).

C. INTRINSIC PROBLEMS

Each of the basic principles on which the success of the Merrifield procedure rests have inborn weaknesses. *Phase separation* results in *slower reac-*

* It was a fortunate coincidence for Merrifield's method that substantial price reductions in commercially available amino acids became effective in the early 1960's due to the development of fermentation processes for their economic production. DCCI also became more readily available at that time.

Table III—Calculated Product Yields of Peptides of Various Chain Lengths for Different Constant Yields per Cycle

Polypeptide, protein	Amino acid residues	Number of cycles	Yield per step			
			95%	98%	99%	99.9%
Angiotensin II	8	7	69.8	86.8	93.2	99.3
Oxytocin, vasopressin, bradykinin	9	8	66.3	85.1	92.3	99.2
Antamanide, gramicidin S	10	9	63.0	83.4	91.4	99.1
α -Melanotropin	13	12	54.0	78.5	88.6	98.8
Gramicidin A	15	14	48.8	75.4	86.9	98.6
Insulin A-Chain	21	20	35.8	66.8	81.8	98.0
Insulin B-Chain	30	29	22.6	55.7	74.7	97.1
Staphylococcal nuclease-P ₈	42	41	12.2	43.7	66.2	96.0
Ferredoxin	55	54	6.3	33.6	58.1	94.7
Bovine trypsin inhibitor	58	57	5.4	31.6	56.4	94.5
Cytochrome c	104	103	0.5	12.4	35.5	90.2
Ribonuclease A	124	123	0.2	8.3	29.1	88.4
Human growth hormone	190	189	0.006	2.2	15.0	82.8

tion, i.e., prolonged reaction periods. *Rapid filtration* requires comparatively large solid particles resulting in *limited loading capacity* in case of surface bonding, or in *diffusion barriers* in case of uniformly distributed bonding throughout the solid support. *One hundred percent quantitative incorporation* of each amino acid residue is impeded by the solid support through additional steric hindrance and possible compartmentalization in the interior of the resin. Even more seriously, the necessary *analytic controls* (to test for complete reaction), which should be of higher sensitivity than usually needed, are considerably *desensitized* by the presence of the solid support. *Complete removal* of the solid support without any degradative effects on the product is difficult to attain. Mild procedures give incomplete removal; strong reagents can cause product degradation. In Sections III and IV various corrective experimental measures shall be discussed which are being applied to counteract these deficiencies to some degree.

What we have to consider for a moment at this time are intrinsic problems arising from the elimination of any intermediate purification throughout a solid-phase synthesis. As a consequence, any side-product resulting from an incident of failure or side reaction is retained in the solid-phase. Repetitive incidents, then, cause accumulation of side-products in numbers that can multiply with increasing numbers of cycles, see Fig. 6. Thus, even very small individual defects can result in serious final deficiencies through the combination of repetition and accumulation.* Faults that can potentially

* The results are well-known from the corresponding financial scheme of compound interest.

Table IV—Theoretical Amounts (in %) of Truncated Sequences Formed in Solid-Phase Synthesis as a Consequence of Constant 10% Chain Termination per Cycle

No. of Cycles: 4th	3rd	2nd	1st	Start
Pro (10)	Pro (10)	Pro (10)	Pro (10)	Pro (100)
Phe-Pro (9)	Phe-Pro (9)	Phe-Pro (9)	Phe-Pro (90)	
Leu-Phe-Pro (8.1)	Leu-Phe-Pro (8.1)	Leu-Phe-Pro (81)		
Orn-Leu-Phe-Pro (7.29)	Orn-Leu-Phe-Pro (72.9)			
Val-Orn-Leu-Phe-Pro (65.6)				

occur in each cycle are (a) permanent chain termination, (b) incomplete reaction, and (c) racemization. Before discussing the effects of these types of incidents on product quality, calculations of overall product yields shall be considered. Table III lists, as a function of different constant yields per cycle, the theoretical amounts of desired target peptides of various chain lengths. Certain biologically active peptides were chosen that have served as target sequences of solid-phase synthesis. The values also apply to solution synthesis by incremental chain elongation (Fig. 2,b). It is obvious from these calculations that for syntheses of proteins containing over 100 amino acid residues an average yield of 99% or more per cycle would be required in order to have any useful amount of the desired target peptide in the product mixture.

The scheme in Table IV shows the effects of formation of so-called "truncated sequences" as a result of permanent chain termination which could, for example, be caused by incomplete deprotection (if the protecting groups remain intact in subsequent cycles). Synthesis of the pentapeptide sequence Val-Orn-Leu-D-Phe-Pro of gramicidin S by incremental chain elongation is taken as an example with an assumed constant 10% chain termination per cycle. The yield of the desired pentapeptide would be 65.6%. Truncated sequences, 7.3% tetrapeptide, 8.1% tripeptide, 9% dipeptide, and 10% starting amino acid, would amount to a total of 34.4%. Since all truncated sequences are retained on the insoluble support throughout the synthesis, the final crude product obtained upon removal of the solid support would be a five-component mixture.

A much more serious problem, however, is the formation of "failure sequences" (Bayer, 1970; Bayer *et al.*, 1970a) or "deletion peptides" (Gutte and Merrifield, 1971) as shown in Table V, again taking a synthesis of Val-Orn-Leu-D-Phe-Pro as an example. Deletion peptides could be formed, e.g., as a consequence of incomplete coupling or of incomplete deprotection and cleavage of remaining protecting groups during the following cycle. With assumed constant 90% coupling yield and 10% formation of deletion peptides per cycle, the final product would contain 65.6% of the target sequence and 34.4% of a contaminant mixture consisting of fifteen different failure sequences (four tetrapeptides amounting to 29.16%, six tripeptides to give a total of 4.86%, four dipeptides adding up to 0.36%, and 0.01% of starting amino acid). The amount of contamination grows in a linear manner, whereas the numbers of individual failure sequences increase according to the binomial equation with growing chain length, thus causing a rapid deterioration of product quality.

The complexity of the "microheterogeneous mixture" caused by deletion peptide formation puts extremely high demands on the fractionation power

Table V.—Theoretical Amounts (in %) of Deletion Peptides Formed in Solid-Phase Synthesis as a Consequence of Constant 90% Yield per Cycle

Cycle Incorporation of:	4th Valine	3rd Ornithine	2nd Leucine	1st D-phenyl- alanine	Start Proline- resin
Failure	Pro (0.01)	Pro (0.1)	Pro (1)	Pro (10)	Pro (100)
	Val-Pro (0.09)				
	Orn-Pro (0.09)	Orn-Pro (0.9)			
	Val-Orn-Pro (0.81)				
	Leu-Pro (0.09)	Leu-Pro (0.9)	Leu-Pro (9)		
	Val-Leu-Pro (0.81)				
Sequences	Orn-Leu-Pro (0.81)	Orn-Leu-Pro (8.1)			
	Val-Orn-Leu-Pro (7.29)				
	Phe-Pro (0.09)	Phe-Pro (0.9)	Phe-Pro (9)	Phe-Pro (90)	
	Val-Phe-Pro (0.81)				
	Orn-Phe-Pro (0.81)	Orn-Phe-Pro (8.1)			
	Val-Orn-Phe-Pro (7.29)				
Target sequence	Leu-Phe-Pro (0.81)	Leu-Phe-Pro (8.1)	Leu-Phe-Pro (81)		
	Val-Leu-Phe-Pro (7.29)				
	Orn-Leu-Phe-Pro (7.29)	Orn-Leu-Phe-Pro (72.9)			
	Val-Orn-Leu-Phe-Pro (65.61)				
Total no. of failure sequences:	15	7	3	1	

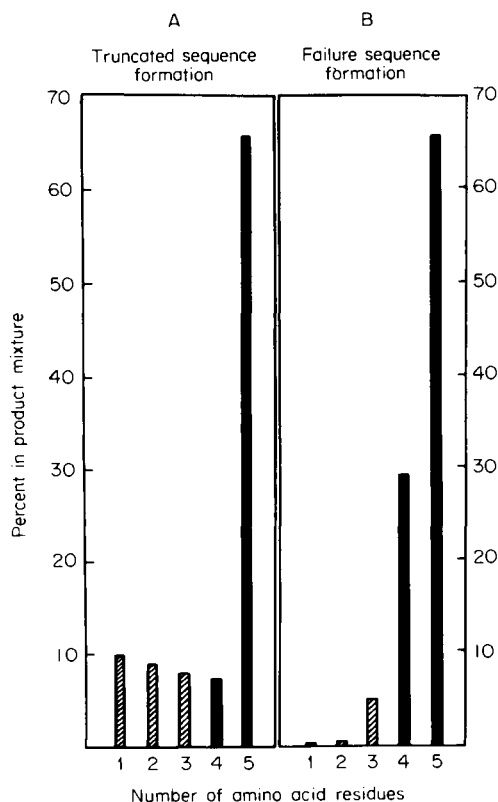


FIG. 5. Relative amounts of contaminating lower peptides (1-4) and target peptide (5) [pentapeptide] in solid-phase synthetic products as a consequence of (A) formation of 10% truncated peptides per cycle, and (B) formation of 10% failure sequence per cycle. Hatched columns show amounts of contaminants (1-3) that are comparatively easy to separate from the target peptide. Solid columns represent mixtures of very closely related peptides (4 + 5) which are difficult to separate.

of purification methods. Microheterogeneity of synthetic peptides is an inherent problem of solid-phase synthesis, as long as reactions do not go to 100% completion throughout all cycles of a given synthesis. An additional complication is the very close relationship of the entire multitude of possible deletion peptides to the target sequence, as shown in Fig. 5B. The major portion of contaminants consists of deletion peptides differing from the target sequence in just one amino acid residue (or in one to several for larger polypeptides or proteins). For peptides larger than deca- or pentadecapeptides, the identification of such closely related failure sequences (each individually present in a small amount) becomes extremely difficult or impos-

sible with presently available analytical procedures. Separation on a preparative scale is even much less feasible, however powerful the fractionation procedures used may be.

By comparison, the major portion of truncated sequences consists of much shorter peptides (Fig. 5A). Their separation from the target sequence should be comparatively easy (e.g., by molecular sieving). Experimental devices to convert potential failure sequence sources into truncated sequences, for example, by termination of unreacted chains through acylation, will be discussed in Section III,D,1.

The relative statistical distribution of peptides in solid-phase synthetic preparations as a function of a constant coupling yield per cycle can be calculated according to the binomial equation, below. After c coupling steps (cycles) giving a yield of $p\%$ each, the theoretical amount A (in percent) of lower chains with n amino acid residues is expressed by:

$$A = 100 \frac{c! \left(\frac{p}{100}\right)^{(n-1)} \cdot \left(1 - \frac{p}{100}\right)^{(c-n+1)}}{(n-1)! \cdot (c-n+1)!}$$

Calculations of failure sequence distribution according to this equation have been tabulated for several peptides by Baas *et al.* (1971). Table VI, for example, shows theoretical values for ribonuclease A. A graphical illustration for several polypeptides and proteins is given in Fig. 6. It becomes evident that a solid-phase synthetic protein preparation, produced according

Table VI—Theoretical Amounts (in %) of Ribonuclease A and of Contaminating Deletion Peptide Mixtures for Various Constant Coupling Yields—123 Coupling Steps; e. g. Ribonuclease A *

Number of amino acids Coupling yield (%)	Mole fraction in % for 123 steps			
	98	99	99.5	99.9
124	8.3	29.0	54.0	88.4
123	20.9	36.1	33.4	10.9
122	26.0	22.2	10.2	0.7
121	21.4	9.1	2.1	
120	13.1	2.7	0.3	
119	6.4	0.7		
118	2.6	0.1		
117	0.9			
116	0.3			
115	0.1			

* From Baas *et al.*, 1971.

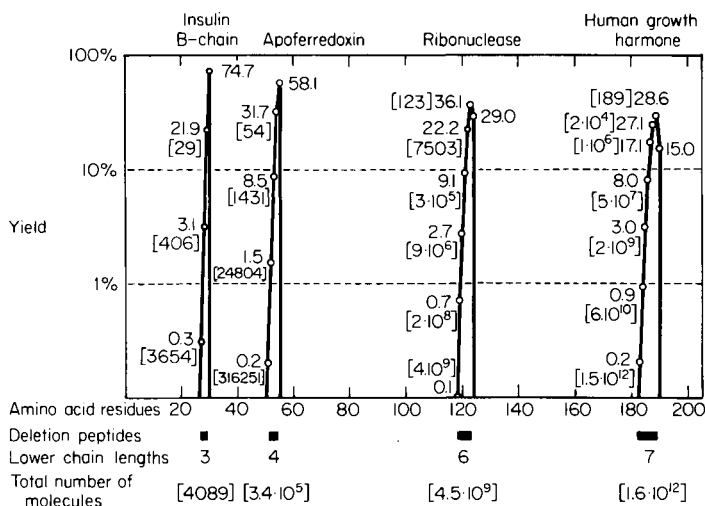


FIG. 6. Theoretical yield of target peptide and of failure sequences assuming a constant 99% yield of amino acid incorporation and 1% of failure peptide formation per cycle. The solid blocks (below the peak areas) designated as deletion peptides and the numerals indicate how many lower chain species contribute to the product mixture in amounts over 0.1%. The numbers of individual failure peptides, in brackets, in these size groups are obtained from the binomial distribution.

to a target sequence of 190 amino acid residues, with an assumed constant 99% yield per cycle, would consist mainly (85%) of an extremely complex mixture of deletion peptides, i.e., 28.6% of chains all possessing 189 residues, 27.1% with 188 residues, 8% having 187 residues, and about 4.2% of the following five shorter chain lengths. The number of different molecules in each category of lower chain length is obtained from the binomial distribution. Thus, the mixture will consist of 189 different molecules possessing 189 residues, of 17,766 molecules with 188 residues, of 1,107,414 molecules with 187 residues, of 51,494,751 molecules with 186 residues, of 1,905,305,787 molecules with 185 residues, of 58,429,377,468 molecules with 184 residues, and of 1,527,510,868,092 molecules with 183 residues. Including the molecule possessing the target sequence, this will add up to 1,587,848,161,467 (approximately 1.6×10^{12}) different molecules in the product mixture, most of which are present only in minute trace amounts. Clearly, yields of over 99.5% or close to 99.9% would be required for solid-phase syntheses of proteins consisting of more than 150 amino acid residues, if one wishes to obtain a respectable mole fraction of the target sequence in the synthetic product. Ultimately, the method should be refined to such a state of perfection that close to quantitative yields can be attained.

Racemization during peptide bond formation can cause the same problem as incomplete reaction. Microheterogeneous mixtures of diastereoisomers accumulate leading to serious deterioration of the optical purity of larger synthetic peptides, even when only 0.1-0.2% racemization per cycle occurs (see Section III,D,1).

The formation of truncated and/or failure sequences during solid-phase synthesis has frequently been played down, and the existence of deletion peptides in the final products has been ignored. Obviously, they cannot, as a rule, be detected individually by such techniques as amino acid analysis, chromatography, or electrophoresis, especially after larger syntheses. Very sensitive tests such as dansyl amino end group determination, seem to have not thus far been applied. Recently a few experimental identifications of truncated or deletion peptides have been published which will be described later (Section III,D,1).

In conclusion, it is clear that the principal operational feature of the solid-phase method, simplification, and tremendous acceleration by abolishing isolation and purification of intermediates does create new problems when reactions are not always 100% complete. Products will be contaminated by very closely related deletion peptides, which for large peptides (>20 amino acid residues) become mostly inseparable from the target sequence. These and other considerations have led to the development of modified solid-phase schemes, which are briefly outlined below.

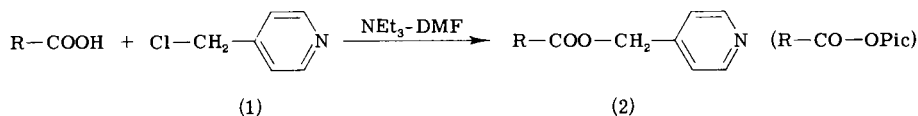
D. RELATED METHODS

1. *Temporary Reversible Attachment of Peptide to Polymer through Ionic Binding*

A scheme to utilize some of the advantages of both the solid-phase procedure (removal of reagents and by-products by simple washing and adsorption) and of solution synthesis (analytic control of coupling reaction and purification of intermediates at every stage) has been developed by G. T. Young and collaborators (Camble *et al.*, 1968; Garner *et al.*, 1968). Reactions are carried out in solution and the protected peptide is then adsorbed on an ion exchanger while the excess of acylating agent and the coproducts are washed away. For this purpose the 4-picolyl ester (2) of the carboxyl terminal amino acid* is utilized as a weakly basic "handle." To attain as complete a coupling as possible, a 2.5-fold excess of N-protected amino acid and of dicyclohexylcarbodiimide or a threefold excess of protected amino acid pentachlorophenyl ester is used relative to the amino acid or

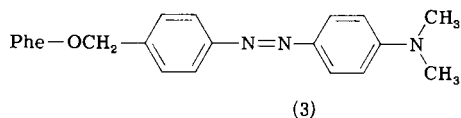
* Benzyloxycarbonylamino acid 4-picolyl esters are prepared by the reaction of the triethylammonium salt of protected amino acids with 4-picolyl chloride (1) in dimethylformamide.

peptide picolyl ester. Coupling reactions are continued until no unchanged amino component can be detected in the reaction mixture by thin-layer chromatography. The protected peptide picolyl ester is isolated from the reaction mixture by adsorption to sulfoethyl Sephadex C-25 (H^+ form), and nonbasic contaminants are washed away with a suitable organic solvent, e.g., tetrahydrofuran. The product is then eluted by means of a solution of triethylamine in an aqueous organic solvent, such as dimethylformamide, dioxane, or tetrahydrofuran. The N-protecting group is removed, and the cycle of operations can be repeated. In this way, chromatographically and analytically pure Leu-Ala-Gly-Val was obtained (Camble *et al.*, 1968, 1969) without any further purification, in 60–65% overall yield. Crude crystalline Z-Pro-Leu-Gly-NH₂ (Garner *et al.*, 1968) was obtained in 52% overall yield and possessed physical constants agreeing with literature data (Ressler and du Vigneaud, 1954). No recrystallization was necessary. The attractive feature of this method is the very high degree of homogeneity of products before any purification; a limitation will very likely be the low solubility of most large protected peptides.



The method has since been modified in that coupling products are generally isolated from reaction mixtures by extraction into 2 *N* citric acid. Adsorption to sulfoethyl Sephadex is used only for those peptide 4-picolyl esters that are insoluble in the aqueous acid (see also: Burton *et al.*, 1971). Syntheses of bradykinin in 42% overall yield using carbodiimide and 2,4,5-trichlorophenyl ester methods for coupling (Schafer *et al.*, 1971; Garner *et al.*, 1971), and of Val⁵-Angiotensin II in 38% yield (Garner and Young, 1969, 1971; Garner *et al.*, 1971) have attested the efficiency of the procedure up to nonapeptide syntheses.

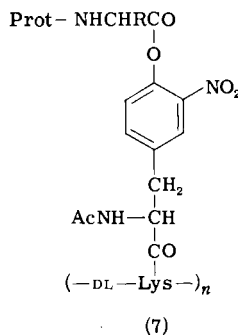
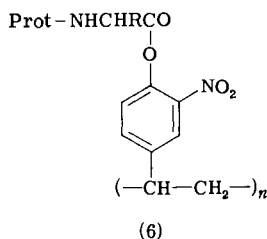
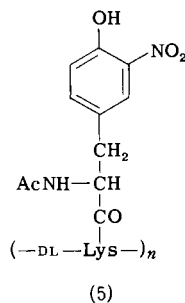
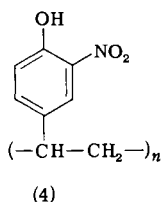
A similar procedure has been described by Wieland and Racky (1968) who used *p*-dimethylaminoazobenzyl phenylalaninate (3) to synthesize Pro-Ala-Phe.



2. Polymeric Reactants or Condensing Agents

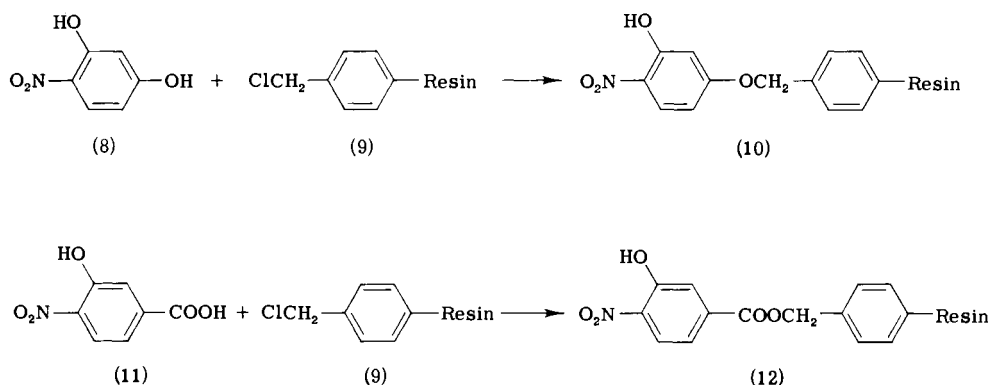
In this solid-phase concept of Katchalski and collaborators (Fridkin *et al.*, 1965b) ["Anti-Merrifield method"] and of Wieland and Birr (1966), the growing peptide chain remains always in solution while the carboxyl com-

ponent constitutes the solid-phase. Copoly-4-hydroxy-3-nitrostyrene-divinylbenzene (4) and copoly-D,L-lysine-3-nitro-L-tyrosine (5) have been used to prepare the respective *o*-nitro-phenyl esters (6 and 7) of N-protected amino acids (Fridkin *et al.*, 1965a,b, 1966a,b; Sklyarov *et al.*, 1966; Patchornik, 1967; Patchornik *et al.*, 1967). Several di- and tripeptides were prepared, and active bradykinin has been synthesized in 39% overall yield (Fridkin *et al.*, 1968) using polymeric esters (6) of benzyloxycarbonyl or *o*-nitrophenylsulfenyl amino acids.



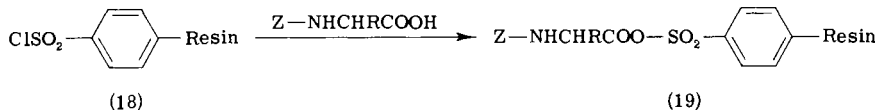
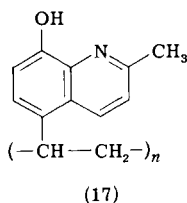
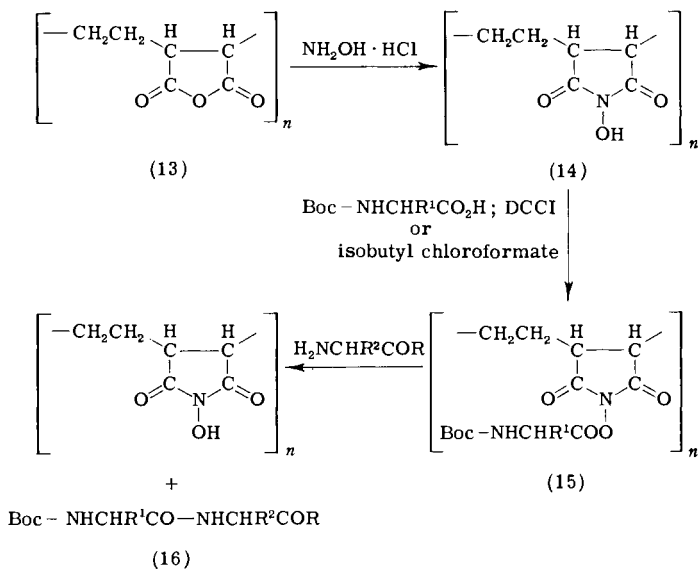
Difficulties observed with these types of polymers led recently to the development of supports (10) in which the active ester function was further away from the polymeric backbone than in (6) (Fridkin *et al.*, 1971). The proven chloromethylated copolystyrene-2% divinylbenzene (9) was used as starting material and condensed with 4-nitroresorcinol (8) to produce solid supports of the *o*-nitrophenol type (10). A similar support (12) has been prepared by Panse and Laufer (1970, 1972) using 3-hydroxy-4-nitrobenzoic acid (11).

Copoly (ethylene-*N*-hydroxymaleimide) (14) obtained from the anhydride (Monsanto Co., St. Louis, Missouri) (13) has been used to prepare

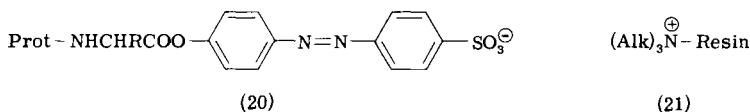


polymeric *N*-hydroxysuccinimide esters (15) in several laboratories (Patchornik *et al.*, 1967; Laufer *et al.*, 1968; Wildi and Johnson, 1968; Akiyama *et al.*, 1969; Fridkin, 1972). A number of small protected peptides (16) have been prepared with the use of (15), as shown below. Similarly, poly(5-vinyl-8-hydroxyquinoline) (17) providing polymeric protected amino acid 8-quinolyl esters (Manecke and Haake, 1968) has been used; and syntheses through polymeric thiophenyl esters have been described (Skylarov *et al.*, 1966; Patchornik *et al.*, 1967). An application of a polymeric mixed anhydride has been reported by Fridkin *et al.* (1971) who prepared the carboxylic-sulfonic anhydride (19) from chlorosulfonylated copolystyrene-2% divinylbenzene (18). Wieland and Birr (1966, 1967b) have described sulfone-activated phenyl esters prepared from a copolymer of *p,p'*-dihydroxydiphenyl sulfone and formaldehyde.

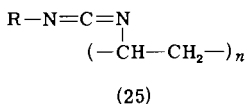
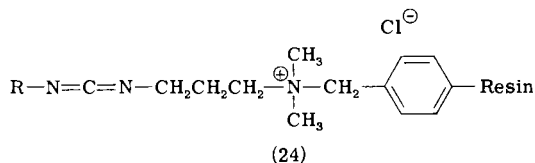
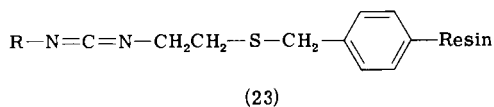
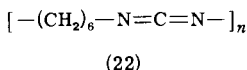
The preparation of the polymeric supports, discussed above, is not always easy. As an alternative, Wieland and Birr (1967a) conveniently attached



active esters of N-protected amino acids, e.g., with 4-hydroxyazobenzene-4'-sulfonic acid (20), by ionic binding to ion exchange resin (21).



The possibility of using carbodiimide in the form of polymeric condensing agents has been investigated by Wolman *et al.* (1967) who prepared polyhexamethylenecarbodiimide (22), and by Fridkin *et al.* (1971) using diimides derived from polystyrene (23, 24) and from polyvinylamine (25) to prepare several di- and tripeptides.



The advantage of these “reverse” procedures lies mainly in the opportunity that intermediates may be purified. However, solubility limitations, diffusion barriers, and/or low reactivity of larger peptides might restrict applications to comparatively small peptides. To date the largest peptides prepared through this strategy have been an octapeptide derivative (Laufer *et al.*, 1968) and bradykinin (Fridkin *et al.*, 1968).

3. Soluble Polymer Supports

To avoid incomplete reactions due to diffusion barriers Shemyakin and collaborators (1965; Kiryushkin *et al.*, 1967) used soluble polymeric supports, e.g., chloromethylated emulsion-polystyrene. The general plan of solid-phase synthesis (Fig. 3) was followed, but the experimental procedure differed. Coupling was carried out by the addition of active esters of Boc-amino acids to dimethylformamide or dioxane solutions of polymer-bound amino acids or peptides. For deprotection anhydrous HCl was bubbled through the mixture. After the coupling step, the peptide-polymer was precipitated by the addition of water. Washing operations to remove excess reactants and by-products were performed on a filter funnel, and the material was ready for the next cycle. Experimental details have been described (Ovchinnikov *et al.*, 1968a), and the conditions necessary for the successful use of the soluble-polymer method have been delineated (Green and Garson, 1969). Care must be taken to block residual chloromethyl groups by acetylation after attachment of the first amino acid residue; otherwise subse-

quent cross-linking insolubilizes the polymer. The method has been used for preparations of Gly-Gly-Leu-Gly (Shemyakin *et al.*, 1965; Ovchinnikov *et al.*, 1968a), of [Gly⁵, Gly¹⁰] gramicidin S (Kiryushkin *et al.*, 1967; Ovchinnikov *et al.*, 1968b), of des(Asp¹, Arg²) Val⁵-angiotensin II (Ovchinnikov *et al.*, 1968b), and of Ala-Ile-Arg (NO₂) Ser-Ala (Green and Garson, 1969), the latter with the use of the carbonic carboxylic anhydride method.

III. The Chemistry

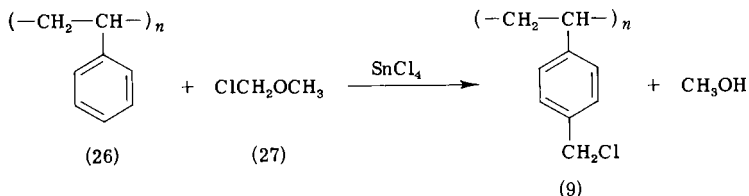
A. THE SOLID SUPPORT AND INTRODUCTION OF FUNCTIONAL GROUPS

1. The Copolystyrene-Divinylbenzene Resin

By far the most successful solid support for peptide synthesis has been a copolymer of styrene and divinylbenzene. This resin is prepared by suspension polymerization in the form of small (200–400 mesh) beads, which measure about 20–80 μ in diameter. It is an amorphous gel with a random network of loosely cross-linked polystyrene chains and fulfills the requirements for an adequate polymeric support (Merrifield, 1963, 1969a). These requirements (Letsinger and Kornet, 1963) include: (1) complete insolubility in all solvents used, (2) chemical and physical stability, (3) a structure and form permitting rapid diffusion of reactants into all reactive sites and easy removal of reactants, by-products, and side products by filtration and washing, and (4) possibility of substitution with a functional group (or possession of a functional group) for the attachment of the first amino acid by a stable covalent bond.

The 2% cross-linked resin, copolystyrene–2% divinylbenzene (26), symbolized as was selected since it appeared to have the most satisfactory properties (Merrifield, 1963), and it has been used since then in the majority of solid-phase peptide syntheses. Chloromethyl groups can readily be introduced into the polymer by treatment with chloromethyl methyl ether (27) and stannous chloride [or another Friedel-Crafts catalyst (Pepper *et al.*, 1953; Gross and Höft, 1967)]. Substitution of every aromatic ring (9) is not desirable, and the reaction can be controlled to provide 0.5–1 meq of chloromethyl groups per gram of resin. This has proven to be a suitable level of functional group concentration for the attachment of the first amino acid.

A number of other supports were investigated during the early days of the solid-phase method. They included rigid copolymers containing large pores fixed by high degrees of cross-linking (Merrifield, 1965b) and popcorn polymers (Letsinger *et al.*, 1964) which do not swell appreciably. These de-



velopments have been reviewed in detail by Merrifield (1969a, pp. 226-233). Many of these other polymers were found to be inferior to the cross-linked polystyrene beads. Progressively decreasing reaction took place with increasing peptide chain length, probably due to diffusion barriers for large reagent molecules and to various other difficulties. Some of these polymers have not been readily available.

Merrifield concluded that the gel-like polymer (26) which swells freely seemed to be a less hindered matrix and a more satisfactory support than rigid polymers. The cross-linked polystyrene-beads are not a "surface support." Due to their swelling in organic solvents they are freely permeable to reagents. Over 99% of Boc-amino acids are located in the interior. Merrifield and Littau (1968) showed by autoradiography of beads containing various tritiated peptides that the distribution is quite uniform throughout the bead (Merrifield, 1969a: Fig. 4). With a substitution level of 0.3 mmole/gm approximately 10^{12} peptide chains are located within a single bead (50 μ diameter). The average space available to each chain amounts to approximately 40 cubic $\text{m}\mu$ (which is enough for a small protein). Another explanation for the success of solid-phase peptide synthesis on the polystyrene resin might be deduced from results of recent kinetic studies. Kemp (1973) observed that peptide bond formation proceeds fastest in toluene as a solvent, both for dicyclohexylcarbodiimide-mediated and for active ester (*N*-hydroxysuccinimide ester) reactions. The polystyrene resin in the swollen gel-like state might resemble a toluene-containing solution.

Because of the importance of resin swelling, knowledge of swelling factors in common solvents is desirable. Table VII gives data determined by Wieland and collaborators (1969a) for a 2% cross-linked polystyrene resin containing approximately 1 mmole/gm of *tert*-butyloxycarbonyl-*L*-phenylalanine residues. Methylene chloride effects the largest swelling and should, therefore, be best suited for syntheses. Dimethylformamide also gives good swelling. This solvent is sometimes required because of its superior dissolving power for many protected amino acid derivatives and reagents. Diethylene glycol dimethyl ether has recently been used (Wieland *et al.*, 1970) for resin swelling prior to attachment of amino acid derivatives.

Reinvestigation of the 1% cross-linked polystyrene (Merrifield, 1969a)

Table VII—Swelling Factors of Boc-Phe-Polymer (Copolystyrene-2% Divinylbenzene) in Various Solvents ^a

Solvent	Swelling factor ^b
Methylene chloride	3.9
Chloroform	3.6
Tetrahydrofuran	3.3
Dioxane	3.2
Dimethylformamide	3.2
Ethyl acetate	2.9
Glacial acetic acid	1.8
Ethanol	1.4
Water	0.95

^a After Wieland *et al.* (1969a).

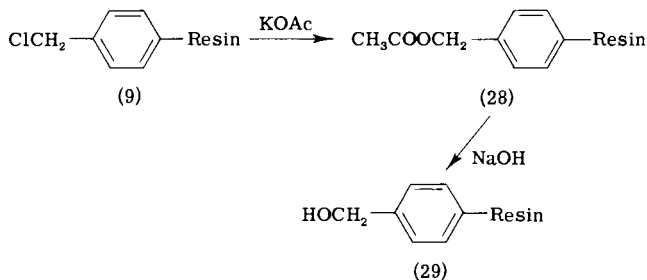
^b Ratio of volume/gm in solvent (after a swelling period of 50 hours at room temperature) to volume/gm of dry resin (1.6 ml/gm).

which initially was reported to be too fragile for continued use over many cycles (Merrifield, 1963), revealed that this support can indeed be used under standard conditions of operation and that it seems to be advantageous in some cases. It swells considerably more in nonpolar solvents; this can result in more complete reactions. Several large peptides and proteins have recently been synthesized on the 1% cross-linked polystyrene support. They include those prepared according to target sequences of bovine ribonuclease A (Gutte and Merrifield, 1969, 1971), human growth hormone (Li and Yamashiro, 1970), *Escherichia coli* acyl carrier protein—(1-74) (Hancock *et al.*, 1971; Marshall *et al.*, 1973) and a partial sequence (Vdovina *et al.*, 1971) as well as five analogs of insulin B-chain (Weber and Weitzel, 1968). However, presently it is not clear whether the rather low quantity obtained in the synthesis of acyl carrier protein was due to loss of solid-phase during filtration as a consequence of breakdown and pulverization of the 1% cross-linked resin (Marshall *et al.*, 1973). The usefulness of a 5% cross-linked polystyrene resin has been examined recently by Orlowska and Drabarek (1971). A high yield of the model peptide Leu-Ala-Gly-Val was readily obtained. In this context, a recent report about a modified procedure for the preparation of cross-linked polystyrene, using methylcellulose as an emulsifier combined with centrifugation, might be of interest (Paruszewski, 1970). Comparative evaluations of different resins would appear to be very desirable. Unfortunately, variable results can even be obtained from repeated syntheses of the same peptide using different batches of the same resin (Bayer *et al.*, 1971b). It has been argued that this finding would offer an explanation for differing results reported in the literature although the reaction conditions had seemingly been the same.

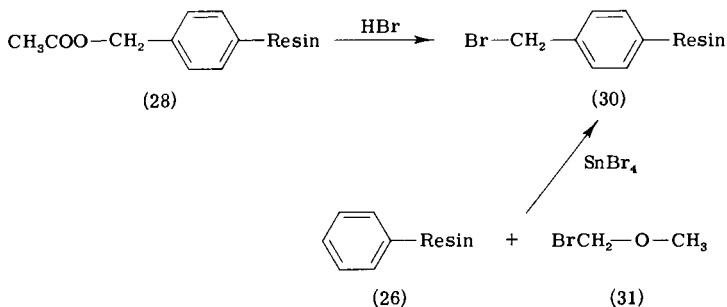
2. Other Supports

Among the many recent methodological studies on the solid-phase method, several have dealt with different solid supports. Some of these have been selected for the purpose of optimizing coupling yields or for allowing milder cleavage of the solid support from the completed peptide. Other supports were sought for the purpose of allowing the use of continuous column procedures. Modified supports can be grouped into those which have been derived from the proven beaded copolystyrene-2% divinylbenzene resin (26) and into those consisting of different polymeric material.

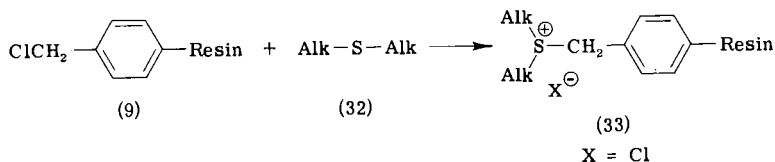
a. Copolystyrene 2% Divinylbenzene Derivatives. A variety of different functional groups have recently been introduced into the standard resin for the purpose of facilitating benzyl ester bond formation. The hydroxymethyl resin (29) was first prepared by Bodanszky and Sheehan (1966) from chloromethylated polymer (9) via the acetoxymethyl resin (28) (compare also, Beyerman and In't Veld, 1969; Meienhofer and Trzeciak, 1971).



Bromomethylated (30) and iodomethylated resins have been produced by treatment of acetoxymethylated material (28) with hydrogen bromide or hydrogen iodide in acetic acid (Tilak, 1968). Bromomethylated resin (30) has also been obtained from cross-linked polystyrene (26) by reaction with bromomethyl methyl ether (31) in the presence of stannic bromide (Yajima *et al.*, 1970c).

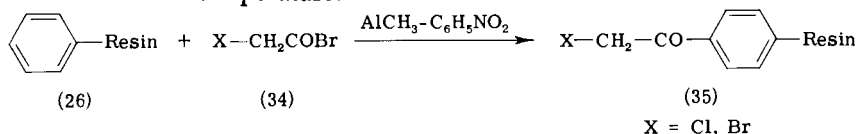


A series of dialkyl(benzyl)sulfonium resins (33) have been prepared by Dorman and Love (1969) through treatment of chloromethylated resin (9) with alkyl sulfides (32). The dimethyl derivative (Alk = CH₃) in the basic

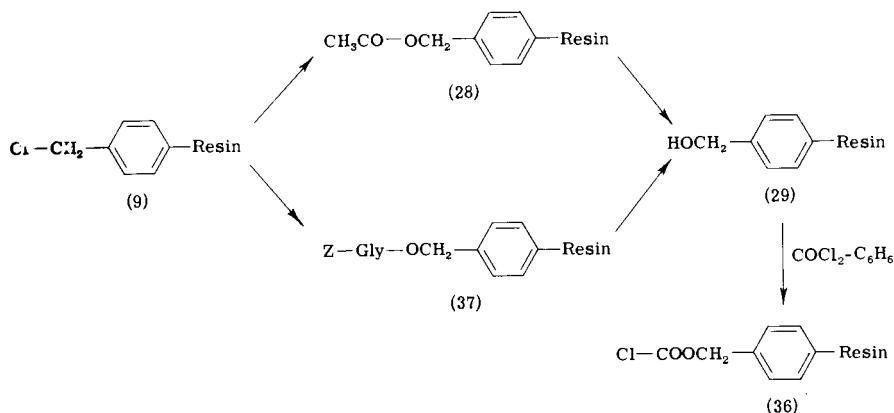


bicarbonate form (X = HCO₃) proved to be most effective for amino acid and peptide attachment.

Modifications of the copolystyrene 2%–divinylbenzene resin (26) involving not only the functional group X but also the anchoring portion A (Fig. 3) between X and the benzene ring moiety provide for the attachment of amino acids or peptides by covalent linkages other than the standard benzyl ester type (Fig. 4). To examine the efficacy of the phenacyl ester peptide to resin attachment, haloacyl polystyrene resins (35) have been prepared (Weygand, 1968; Mizoguchi *et al.*, 1969, 1970). Best results were obtained when copolystyrene–2% divinylbenzene (26), swelled in nitrobenzene, was treated with 0.4 equiv of haloacetyl bromide (34) and aluminum chloride at room temperature.

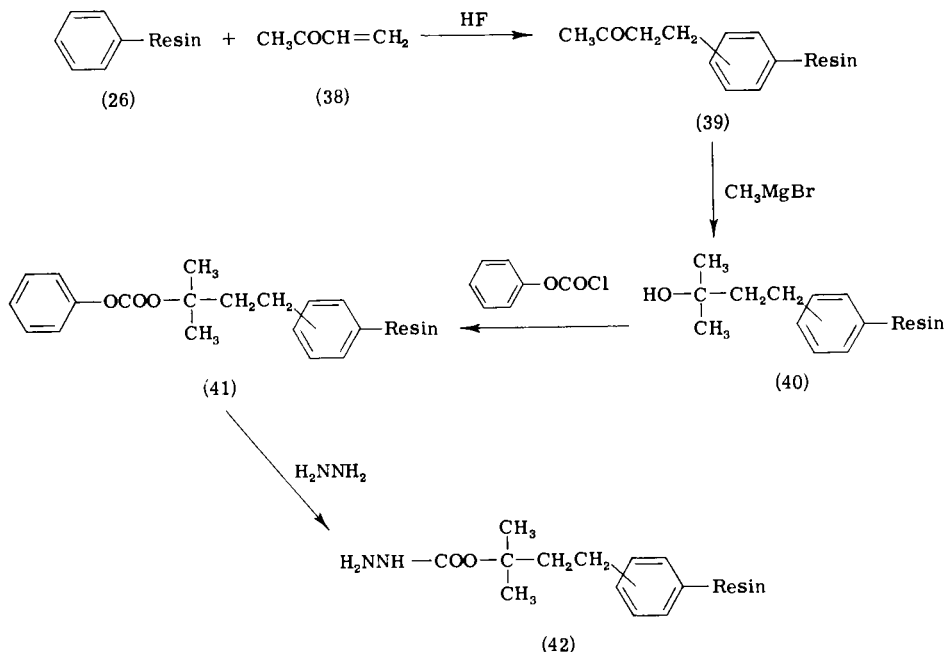


Chloroformoxymethyl group-bearing popcorn polymers were first used by Letsinger and Kornet (1963). The introduction of the group into polystyrene resin (36) has been carried out via the acetoxymethyl (28) and hy-

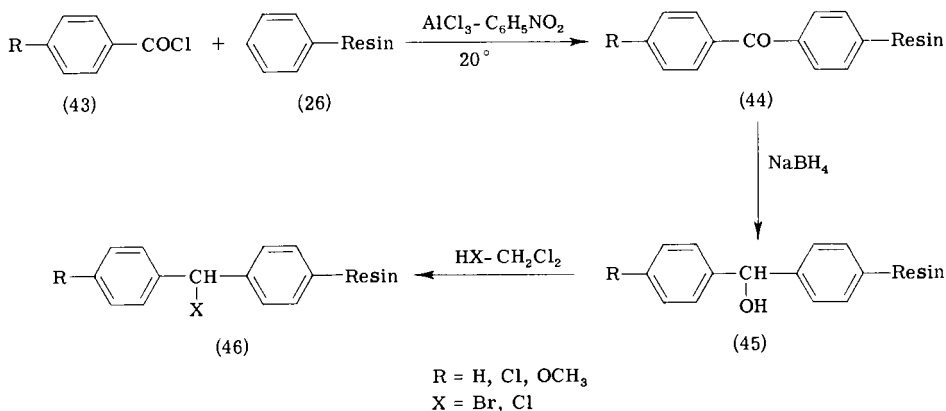


droxymethyl (29) intermediates (Felix and Merrifield, 1970; Meienhofer and Trzeciak, 1971) or via *Z*-glycine-resin (37) (Sklyarov and Shashkova, 1969).

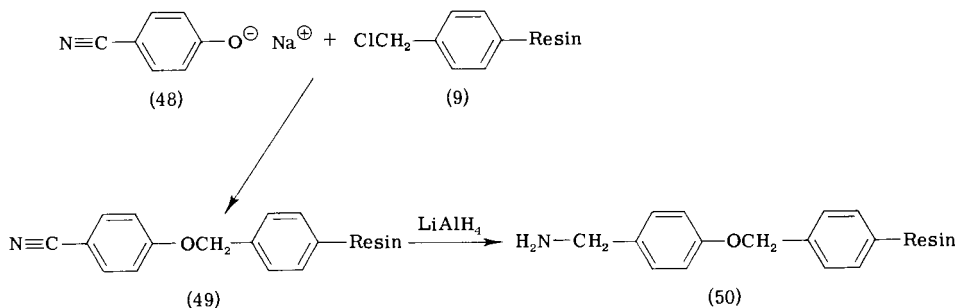
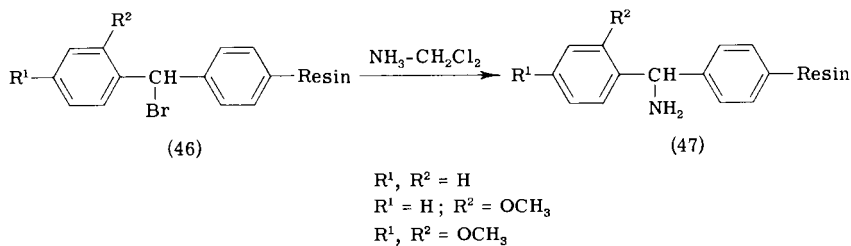
A series of reactions leading to both *tert*-alkyl alcohol (40) and to *tert*-alkyloxycarbonylhydrazine substituted resins (42) has been devised by Wang and Merrifield (1969a). Reaction of copolystyrene-2% divinylbenzene (26) with methyl vinyl ketone (38) in the presence of HF to give 3-oxobutyl resin (39) is followed by a Grignard reaction which provides the *tert*-amyl alcohol resin (40). For the preparation of the *tert*-alkyloxycarbonyl hydrazide resin (42), the alcohol resin (40) is reacted with phenyl chloroformate to form a phenyl carbonate derivative (41) which is then hydrazinolized.



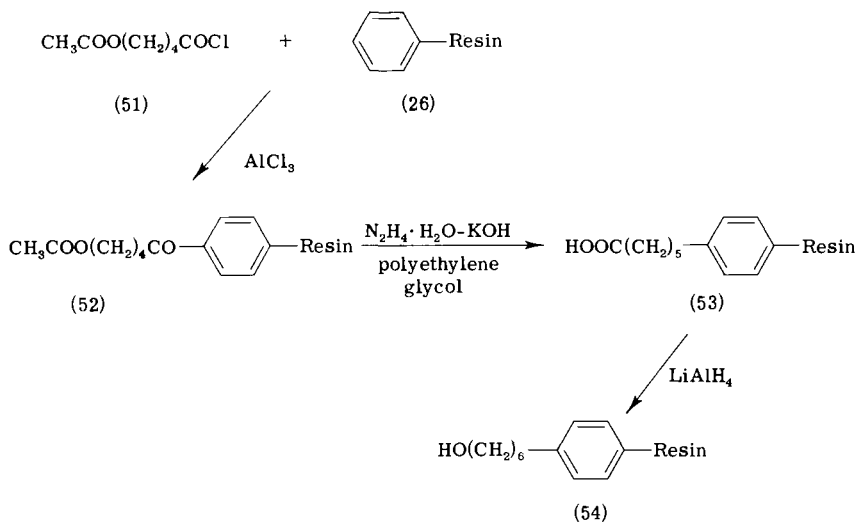
Benzhydryl polystyrene resins (46) have also been prepared in efforts to attain milder removal conditions at the end of solid-phase syntheses (Southard *et al.*, 1969, 1971b,c). Friedel-Crafts condensation of copolystyrene-2% divinylbenzene (26) with benzoyl chloride (43, R = H) affords (44) which is reduced to the carbinol (45) by sodium borohydride. Conversion to the desired benzhydryl halide resins (46) is effected by treatment with anhydrous HBr or HCl in methylene chloride. Both the *p*-chloro- and *p*-methoxybenzhydryl resins have been prepared similarly.



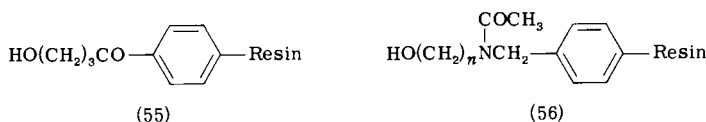
Benzhydrylamine (47) and *p*-methoxybenzylamine (50) resins offer peptide resin attachment through amide bonds and provide peptide amides upon treatment with HF. Benzhydrylamine resins are obtained by treatment of (46) with ammonia in methylene chloride (Pietta and Marshall, 1970; Pietta *et al.*, 1973). The *p*-methoxybenzylamine resin (50) is prepared by reaction of sodium *p*-cyanophenoxide (48) with chloromethylated copoly-styrene-2% divinylbenzene, followed by reduction of (49) with lithium aluminum hydride (Pietta and Marshall, 1970).



The 6-hydroxy-*n*-hexyl resin (54) has been reported (Bayer *et al.*, 1971a) to provide for facilitated aminolysis of peptides with sterically hindered C-terminal amino acids, because the long anchoring chain places the peptide to resin link farther away from the polymer backbone, thus reducing steric hindrance. The support (54) is obtained by Friedel-Crafts condensation of copolystyrene-2% divinylbenzene (26) with the monomethyl ester of adipic acid monochloride (51) followed by reductions of the product (52) first by a Wolff-Kishner reaction to give (53) and then by lithium aluminum hydride.



Other supports possessing alkanol functions (55, 56) had previously been described by Tilak and Hollinden (1968a,b).

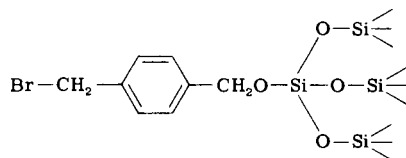


So-called "dual function" or "safety catch" supports provide peptide to resin linkages that can be converted at the end of a synthesis into a reactive bond for easy removal of the solid support or for cyclization on the support. A resin (58) containing the 4-methylthiophenyl group of Johnson and Jacobs (1968a,b) has been obtained by refluxing chloromethylated copolystyrene-2% divinylbenzene (9) and *p*-mercaptophenol (57) in dimethylformamide (Flanigan and Marshall, 1970, 1972; Marshall and Lie-ner, 1970).

standard Merrifield-resin. A similar macroporous polystyrene support, beaded SM-1 resin (supplied by Calbiochem, Los Angeles, California), with average pore size of 200 Å, has been investigated by Tilak and Hollinden (1971). After chloromethylation, homogeneous Ala-Phe was prepared by standard procedures. The resin was also derivatized as in (56) and a tetrapeptide was then synthesized.

Graft-polymer resins have been developed for solid-phase synthesis by Potts and Tregear. Styrene is polymerized onto small Teflon or Kel-F beads with the aid of γ -radiation (Tregear *et al.*, 1967). A poly-(trifluorochloroethylene)-*g*-chloromethylstyrene containing about 7% polystyrene was used for synthesis of the N-terminal tetratriacontapeptide of bovine parathyroid hormone (Potts *et al.*, 1971) and for a synthesis of substance P (Tregear *et al.*, 1971). The apparent rationale for the development of grafted polymers for solid-phase synthesis is an assumed improvement in attaining both more complete and faster reactions in the incorporation of each amino acid residue by reducing diffusion into the interior of the resin. Another approach to minimize diffusion was the use of pellicular resins (Bayer *et al.*, 1970a) which were prepared by polymerizing a thin layer of resin onto inert glass beads (Horvath *et al.*, 1967). Considerable improvements in eliminating the occurrence of failure sequences in (Leu-Ala)₆ were observed with the use of pellicular resin supports, but limited mechanical stability due to peeling of the resin from the glass (Bayer *et al.*, 1971b) has discouraged further use.

In attempts to simplify technical aspects of solid-phase synthesis by the use of column procedures, which are not feasible for swelling resins, Bayer and collaborators (1970c, 1971b; Parr and Grohmann, 1971) proposed the application of porous glass, silica gel, or quartz as supports. Alcohols can be esterified to silanol groups on the surface of silica (Halász and Sebastian, 1969) providing locations for functional groups purely on the surface of the inorganic support (62). Since these organic groups are con-



(62)

sidered to protrude from the surface, the polymer resembles a brush having the functional group for amino acid attachment at the end of the bristle; therefore, the term "brush-type" support has been coined.* Syntheses of

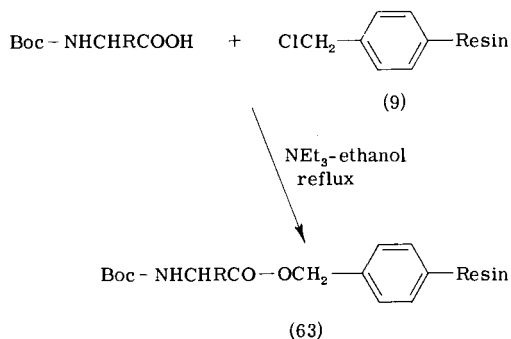
* Materials of this type are available under the name of Biopak from Waters Associates, Framingham, Ma.

(Leu-Ala)₆ and of Leu-Leu-Gln-Gly [Secretin-(22-25)] in a specially designed synthesizer for column operation showed that reaction times were considerably shorter than those required with the polystyrene resin in the standard rocking vessel. Further advantages claimed for column operation over batch procedures include: easier scaling up; elimination of shaking so there is no mechanical corrosion of the solid-phase; and smaller excesses of reagents to obtain comparable equilibria. Both the brush support and the graft polymer have several promising features. However, it is still too early to evaluate their relative merits or potentials as well as those of other newly developed supports discussed above.

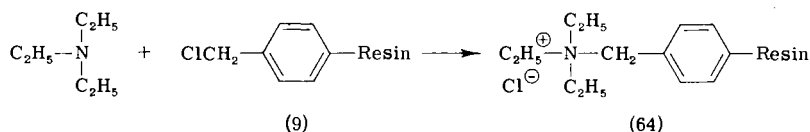
B. ATTACHMENT OF AMINO ACIDS OR PEPTIDES

1. The Standard Benzyl Ester Linkage

Benzyl ester bond attachment (63) of the first amino acid through reaction with chloromethylated copolystyrene-2% divinylbenzene has been used for the great majority of all syntheses to date (see Merrifield, 1969a; Stewart and Young, 1969; Marshall and Merrifield, 1970). In general, *tert*-butyloxycarbonylamino acids and chloromethylated resin (9) are incubated



for 24-50 hours in the presence of triethylamine in refluxing ethanol or other solvents (For a detailed study, see Losse *et al.*, 1968b). To avoid competing benzyl ether formation or transesterification (Halpern *et al.*, 1968; Beyerman *et al.*, 1968b), tetrahydrofuran was recommended as a solvent (Wieland *et al.*, 1969a). No quaternization, i.e., formation of resin-bound triethyl benzyl ammonium groups (64), was observed with the use of tetra-

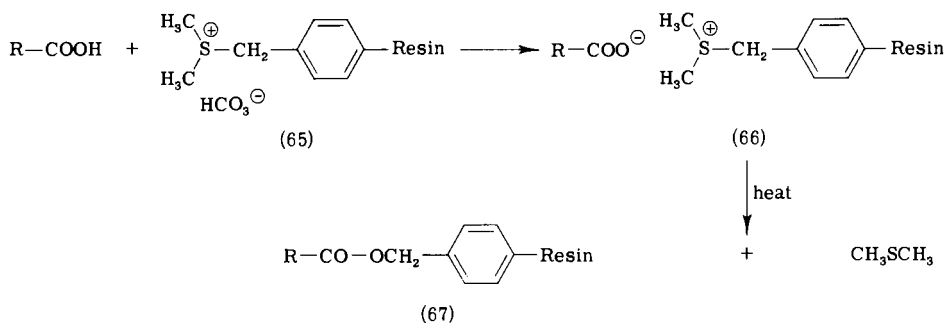


hydrofuran, whereas this side reaction occurs to considerable degrees in ethanol (Rudinger and Gut, 1967). The use of tetramethylammonium hydroxide instead of triethylamine for the attachment reaction has been described by Loffet (1971). Better yields were obtained and no quaternary ammonium derivatives were formed on the resin. Alternatively, the application of a mixture of sodium carbonate and dimethylformamide has provided very satisfactory esterification of Boc-amino acids to resins (Green and Garson, 1969; Beyerman *et al.*, 1973a).

Some amino acid derivatives, such as *o*-nitrophenylsulfenylamino acids or *tert*-amyloxycarbonylmethionine, are unstable at the commonly used high-temperature esterification conditions, as reported by Losse and Neubert (1968b) and Inukai *et al.* (1968), respectively. Alternative milder procedures of benzyl ester bond formation have therefore been proposed (Merrifield, 1969a). Using dimethylformamide as a solvent, Marglin (1971) obtained resin substitution (0.1–0.3 meq/gm) at room temperature within 24 hours by treating 1 gm of chloromethyl resin with 1 mmole of Boc-amino acid and 0.9 mmole of triethylamine. A strategem to attach methionine to resin has been suggested by Norris *et al.* (1971). Boc-methionine sulfoxide can be treated in the standard manner of using triethylamine, ethanol, or ethyl acetate, 24-hour reflux, under nitrogen in the dark. Conversion (98%) to Boc-methionine resin is affected by reduction with sodium iodide–acetyl chloride in dimethylformamide.

Bodanszky and Sheehan (1966) pioneered the use of the hydroxymethyl resin (29). Attachment of protected amino acids to this resin is effected under mild conditions by *N,N'*-carbonyldiimidazole (Staab and Wendel, 1963; Losse and Klengel, 1971), by dicyclohexylcarbodiimide in pyridine (Henecka, 1952; Rothe and Kunitz, 1957), and by *N,N'*-dimethylformamide dineopentylacetal (Büchi *et al.*, 1963; Schreiber, 1967). An elegant procedure of attachment was developed by Dorman and Love (1969). Basic dimethylsulfonium styrene–2% divinylbenzene resin in the bicarbonate form (65) provides for convenient, efficient, and rapid attachment of amino acid derivatives (see, also, Dorman and Markley, 1971). Irreversible binding via salt formation (66) between the carboxyl group and the sulfonium resin is followed by warming in the dry state to effect a displacement reaction (66→67). The procedure is mild enough to allow the attachment of *o*-nitrophenyl-sulfenylamino acids to the resin. *N*-Protected peptides (up to nonapeptides) and even *N*-unprotected peptides have been linked to solid supports through this type of functional group (65) (Fontana *et al.*, 1971).

Bromomethylated (30) and iodomethylated resins (Tilak, 1968) allow benzyl ester formation under considerably milder conditions than those re-



quired for the chloromethylated resin. The bromo and iodo derivatives react within a few hours at room temperature with *N*-protected amino acids in the presence of base. *N* α -*tert*-butyloxycarbonyl-, *o*-nitrophenylsulfonyl-, and 1,3-diketone enamine protected amino acids have been successfully esterified to these solid supports. The tetrapeptide Boc-Lys(Z)-Pro-Val-Gly-OH was attached to a bromomethyl resin in the presence of dicyclohexylamine (Yajima *et al.*, 1970c). Vesa and Dirvianskyte (1968) reported that it was superfluous to prepare the iodomethylated resin separately. The attachment reaction can be performed in one step using chloromethylated resin and sodium iodide as a catalyst. Although the modified procedures for improved resin-benzyl ester bond formation have indeed enhanced the scope of the procedure, the majority of amino acid attachments are still being carried out according to the original method.

In spite of its numerous applications "the original benzyl ester-type linkage is perhaps one of the least satisfactory features of the Merrifield synthesis" (Sheppard, 1973). The main disadvantage lies in the drastic conditions (hydrogen bromide in trifluoroacetic acid or liquid anhydrous hydrogen fluoride) required for its cleavage at the end of a synthesis. These conditions can lead to considerable degradation of the desired peptide, and at the same time can still leave the cleavage of the bond to the solid support incomplete. These aspects will be discussed in detail later (Section III,E,1). Another shortcoming of the benzyl ester linkage lies in its incomplete stability to the deprotecting agents, especially trifluoroacetic acid (Nitecki and Goodman, 1970). Small amounts are progressively cleaved during the course of the synthesis, resulting in loss of peptide from the resin and a lowering of the overall yield. Gutte and Merrifield (1969) calculated that the total loss of material through 123 cycles of a synthesis (target sequence of ribonuclease A) amounted to an average of 1.4% loss per cycle. A lower loss of approximately 0.4% of Boc-methionine sulfoxide per (simulated) cycle was determined by Norris *et al.* (1971) over thirty-two repeated treatments by 1 *N*

HCl in acetic acid followed by triethylamine in methylene chloride. Originally Merrifield (1962, 1963) used a nitrated resin because nitrobenzyl esters are known to be more stable to acid than benzyl esters. Since the acidolytic removal of the solid support from the completed peptide is thereby also impeded, nitrated resins have seldom been used (e.g. Takashima *et al.*, 1968; Arai *et al.*, 1970).

2. Alternative Types of Linkages

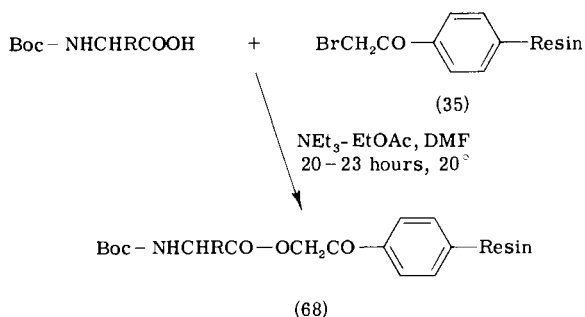
a. Through α Functional Groups. The development of alternative types of bonds has received little attention until recently. The requirements for an ideal linkage of peptide to solid support are very demanding. The covalent bond has to be readily formed, completely stable throughout the repetitive cycles of synthesis, and yet be subject to cleavage under mild conditions at the end of the synthesis. To arrive at an ideal linkage serving all purposes of solid-phase peptide synthesis might perhaps require a prolonged search. In the interim, an arsenal of several less perfect supports with widely varying types of functional groups for attachment might be useful, each serving a specific purpose. Objectives which could be better met by other linkages than a benzyl ester include:

- i.* Removal from the resin under mild conditions compatible with the stability of the peptide;
- ii.* Preparation of protected peptides required as intermediates for fragment condensation, and
- iii.* Complete stability of peptide-resin bond throughout the synthesis.

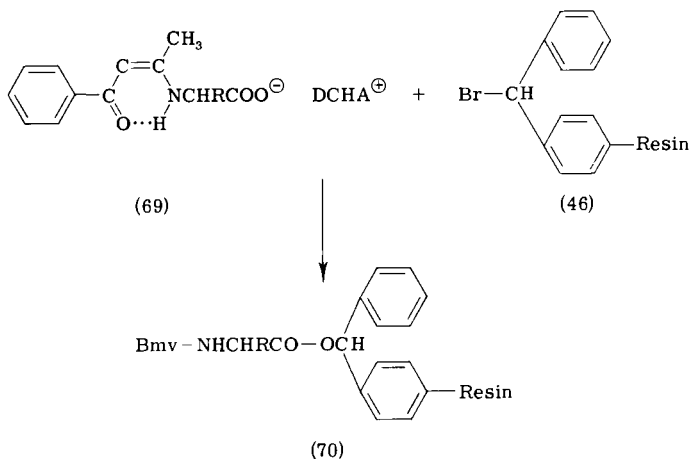
Mild removal conditions (i) for the completed peptide are provided through the phenacyl ester linkage (sodium thiophenoxide, NH_3 , N_2H_4), through the benzydryl and *tert*-alkyl ester linkages (5–50% trifluoroacetic acid in chloroform or methylene chloride), through the peptide linkage (proteolytic enzymes), through the methylthiophenyl ester linkage (base, NH_3 , N_2H_4 , after oxidation), and through the acylsulfonamide linkage (base, after N-methylation). The preparation of the solid supports required for these types of peptide-resin linkage have been described in the preceding section (Section III,A,2). The attachment reactions are outlined below.

Phenacyl ester bonds (68) have best been obtained (Weygand, 1968; Mizoguchi *et al.*, 1969, 1970) by reacting bromoacetyl polystyrene (35, X = Br) with, e.g., Boc-amino acids, for 20–23 hours at room temperature in the presence of an equivalent of *tertiary* amine, using ethyl acetate or dimethylformamide as solvents.

To obtain benzydryl ester linkages (70) Southard *et al.* (1969, 1971c) treated phenylbromomethylstyrene–2% divinylbenzene copolymer resin (46, X = Br, R = H) with 2-benzoyl-1-methylvinyl (Bmv) amino acid



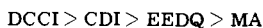
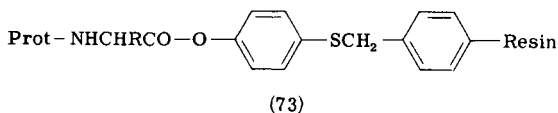
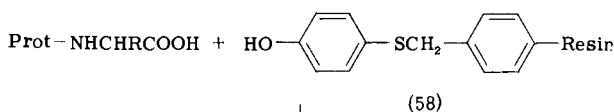
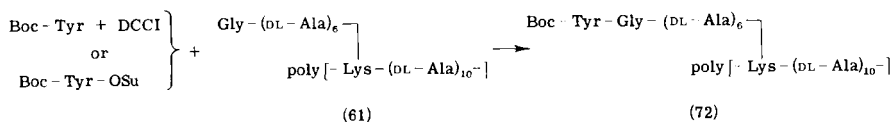
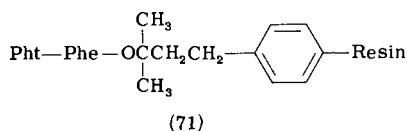
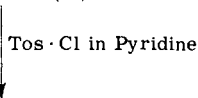
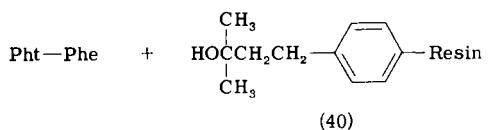
dicyclohexylammonium salts (69) either for 4 days at room temperature in methylene chloride, or for 15 hours in refluxing chloroform.



A mixed anhydride reaction has been used to obtain *tert*-alkyl ester bonds (71) (Wang and Merrifield, 1971). The alcohol resin (40) was reacted with phthalyl-L-phenylalanine (1 equiv) and toluenesulfonyl chloride (2 equiv) in dry pyridine at 0° for 1 hour and then for 24 hours at 4°.

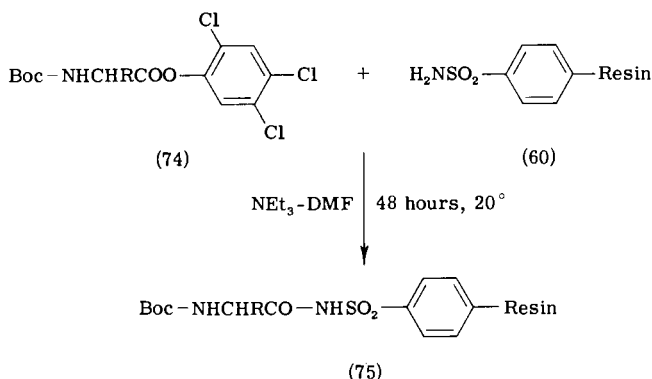
Attachment of Boc-amino acids to multichain polyamino acid supports (61) was effected (72) by dicyclohexylcarbodiimide or through Boc-amino acid *N*-hydroxysuccinimide esters (Anfinsen, 1968).

Methylthiophenyl ester linkages (73) have been accessible through condensation of protected amino acids with methylthiophenol-polystyrene (58) using dicyclohexylcarbodiimide, carbonyldiimidazole, mixed anhydride (MA) or *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ) in methylene chloride or tetrahydrofuran as solvents. When equimolar amounts of reactants were used, the condensing efficiency to give (73) was as follows: DCCI > CDI > EEDQ > MA (Flanigan and Marshall, 1970, 1972).



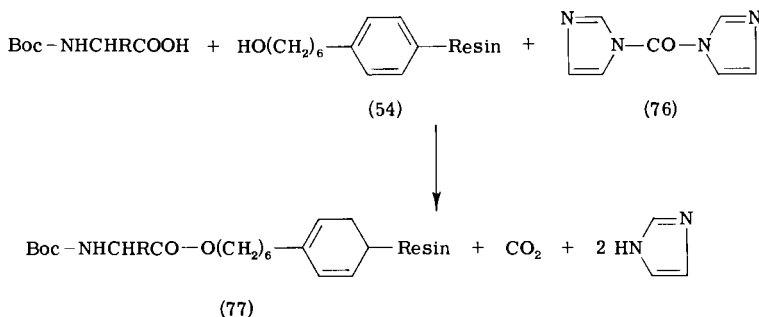
The formation of acylsulfonamide bonds (75) was achieved (Kenner *et al.*, 1971) by treatment of the sulfonamide resin (60) in dimethylformamide with Boc-amino acid 2,4,5-trichlorophenyl esters (74) in the presence of triethylamine (5 equiv) for 48 hours at room temperature.

Protected peptides (ii), e.g., amides and hydrazides, are prepared by direct ammonolysis or hydrazinolysis of the standard benzyl ester peptide to resin linkage (see Section III,E,1). Hydrazides are useful as intermediates for fragment condensation by the azide method. Alternative linkages allowing ammonolysis or hydrazinolysis include the alkyl ester bonding to Sephadex supports (Vlasov and Bilibin, 1969) or to the modified polystyrene supports (55, 56) described by Tilak and Hollinden (1968a,b). Facilitated (accelerated) amide and hydrazide formation was obtained through a variety of bonds, e.g. phenacyl esters (Mizoguchi *et al.*, 1969, 1970), phenyl esters (Inukai *et*

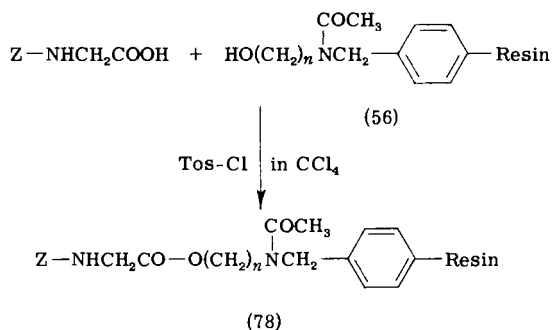


al., 1968), long-chain ω -alkyl esters [by virtue of diminished steric hindrance (Bayer *et al.*, 1971a)], and methylthiophenyl esters (73) (Flanigan and Marshall, 1970) or acylsulfonamides (75) (Kenner *et al.*, 1971) after activation (see below). Peptides containing side-chain benzyl esters cannot be subjected to ammonolysis or hydrazinolysis. To make hydrazides of these peptides available, Wang and Merrifield (1969a) used the *tert*-butyloxycarbonyl hydrazide peptide to resin linkage in combination with the *N* α -2-(*p*-biphenyl)isopropoxyloxycarbonyl protection. Hydrazides are obtained by mild acidolysis (e.g., 50% trifluoroacetic acid in methylene chloride). The benzhydrylamine resin, developed by Pietta and Marshall (1970; see, also, Pietta *et al.*, 1973), gives peptide amides when treated with hydrogen fluoride. Attachment reactions to form phenacylestere (68), methylthiophenyl esters (73), and acylsulfonamides (75) have been described above.

Alkyl ester linkages between protected amino acids and solid supports have mostly been obtained with the aid of carbonyldiimidazole (Staab and Wendel, 1963). For example, treatment of 6-hydroxy-*n*-hexylstyrene-2% divinylbenzene (54) with equimolar amounts of Boc-amino acid and carbonyldiimidazole (76) in methylene chloride at room temperature gave (77).

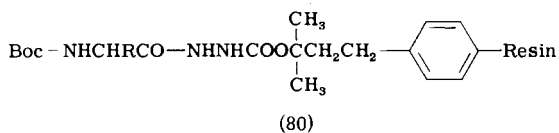
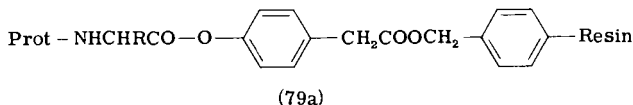
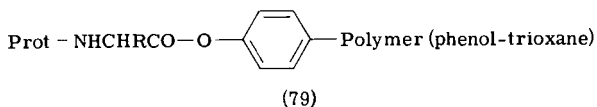


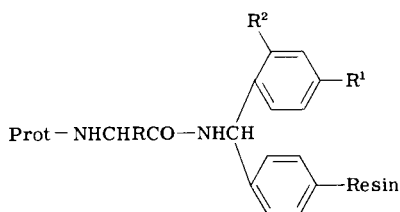
In addition, a mixed anhydride reaction has been employed to attach Boc-amino acids to Sephadex supports (Vlasov and Bilibin, 1969) and *p*-toluenesulfonyl chloride served to form an alkyl ester bonding (78) (Buka and Zagats, 1969) between Z-glycine and resin (56).



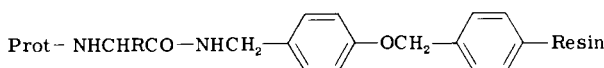
Phenyl esters (79) have been formed with the aid of dicyclohexylcarbodiimide (Inukai *et al.*, 1968) or with *N,N'*-carbonyldiimidazole to give (79a) (Blake and Li, 1971). The same reagents served to attach (80) protected amino acids to the *tert*-alkyloxycarbonyl-hydrazine resin (Wang and Merrifield, 1969a) and to form benzhydrylamide (81) (Pietta and Marshall, 1970; Pietta *et al.*, 1972) or *p*-methoxybenzyl-amide linkages (82) (Pietta and Marshall, 1970).

Complete stability of the bonds between peptide and support (iii) throughout the synthesis and the use of mild conditions for the cleavage of





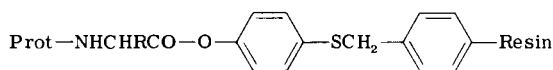
(81)

 $\text{R}^1, \text{R}^2 = \text{H}, \text{OCH}_3$ 

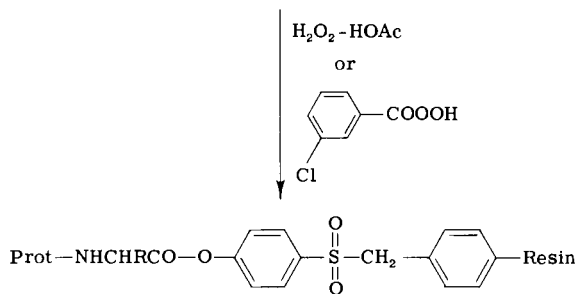
(82)

these same bonds at the end of the synthesis are somewhat contradictory requirements. One way to solve the dilemma is to change the nature of the bonding by chemical modification prior to the cleavage, a design that has been named the "safety catch" principle (Rudinger, 1963). The 4-methylthio)phenyl esters (Johnson and Jakobs, 1968a,b; Johnson and Trask, 1968) provide a very stable linkage (73) which can be oxidatively converted into an activated ester group [4-(methylsulfonyl)phenyl ester: Iselin *et al.*, 1957; Schwyzer and Sieber, 1958] with the aid of hydrogen peroxide in acetic acid or of *m*-chloroperbenzoic acid in dioxane (Flanigan and Marshall, 1970, 1972).

Another system featuring oxidative conversion has been described by Wieland *et al.* (1970) based on the known method of peptide bond forma-

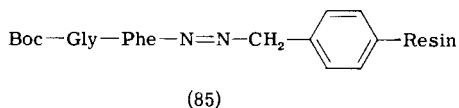
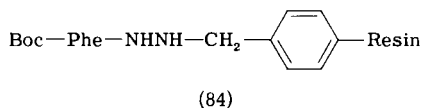


(73)

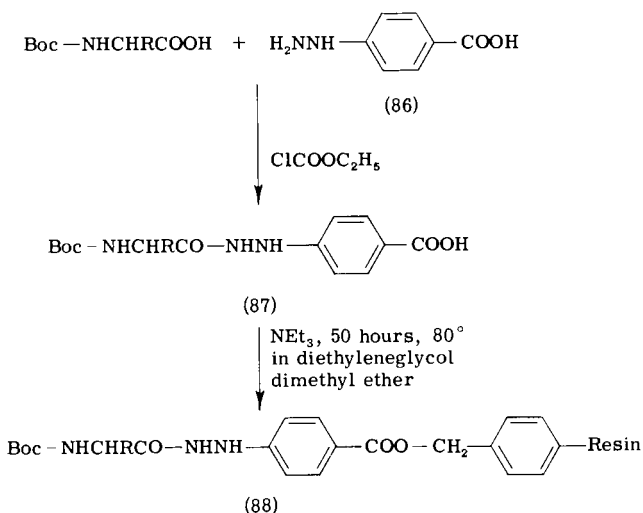


(83)

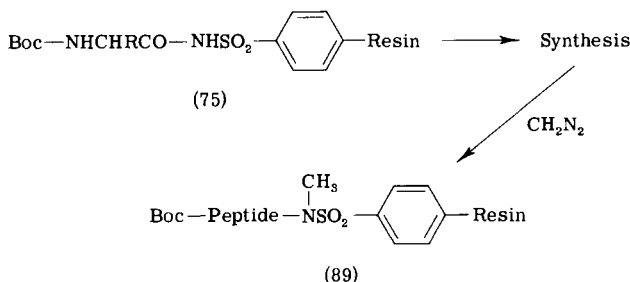
tion through the oxidative activation of acylamino acid hydrazides (Wolman *et al.*, 1961, 1962) or phenylhydrazides (Milne and Kilday, 1965; Milne and Most, 1968). Thus, *tert*-butyloxycarbonylphenylalanine hydrazide was linked to the Merrifield resin by incubation for 50 hours at 80° in the presence of triethylamine using diethylene glycol dimethyl ether as a solvent, to give a very stable linkage to the solid support (84). *N*-Bromosuccinimide oxidation in pyridine, after completion of the synthesis, produced the reactive aliphatic azofunction (85) which could be readily cleaved by aminolysis or hydrolysis. The danger of undesired spontaneous decomposition of



(85) led to the development of an aromatic system. 4-Hydrazinobenzoic acid (86) gave, by a mixed anhydride condensation, acylamino acid 4-carboxyphenylhydrazides (87) which were then attached (88) to chloromethylated cross-linked polystyrene as described for (84). This stable attachment proved to be useful for the synthesis of a decapeptide, Phe-Pro-Pro-Phe-Phe-Val-Pro-Pro-Ala-Phe (linear antamanide precursor), which was obtained with excellent yield.



The oxidative methods impose, however, unfortunate restrictions and will be of no use for the synthesis of peptides containing cysteine, cystine, methionine, tyrosine, and tryptophan residues. A nonoxidative safety catch system has been applied to solid-phase synthesis by Kenner *et al.* (1971). The acylsulfonamide linkage (75) was found to be completely stable to the conditions of solid-phase synthesis, especially to the acidolytic deprotection reaction. N-Methylation (89) by diazomethane after completion of the synthesis allowed to use mild alkaline cleavage* (0.5 N NaOH), ammonolysis, or hydrazinolysis. Satisfactory results were obtained with this system (Sheppard, 1973), e.g., in the preparation of the C-terminal octapeptide of calcitonin M (Kenner *et al.*, 1971).



b. Through Side-Chain Functional Groups. In all the investigations described above, variation of the peptide to resin linkage was attained through the use of different functional groups on the resin. Amino acids or peptides have always been attached through the α functional groups, i.e., in almost all cases the α -carboxyl group. We have suggested (Meienhofer and Trzeciak, 1971) that the scope of solid-phase peptide synthesis could also be broadened by utilizing the large variety of functional groups in amino acid side-chains for attachment to solid supports. These tactics offer possibilities such as: (a) disulfide linkages between the thiol functions of cysteine or homocysteine residues and thiol-containing resins, (b) ester linkages between the hydroxyl groups of serine, threonine, or tyrosine and resins carrying carboxyl groups, (c) ether linkages between the hydroxyl amino acids and solid supports, (d) ester linkages between the ω -carboxyl groups of aspartic or glutamic acid residues and hydroxyl-containing resins, (e) amide linkages between the ω -carboxamido groups of asparagine or glutamine and benzhydryl resins and (f) urethane linkages between the ω -amino functions of the basic amino acids (lysine, ornithine, diaminobutyric acid, arginine) and

* This was based on earlier observations (Rudinger, 1963; Wieland and Hennig, 1960) concerning the stability of acylsulfonamides to basic reagents (because of ionization of the nitrogen, $pK_a \sim 2.5$) and their ready cleavage after methylation.

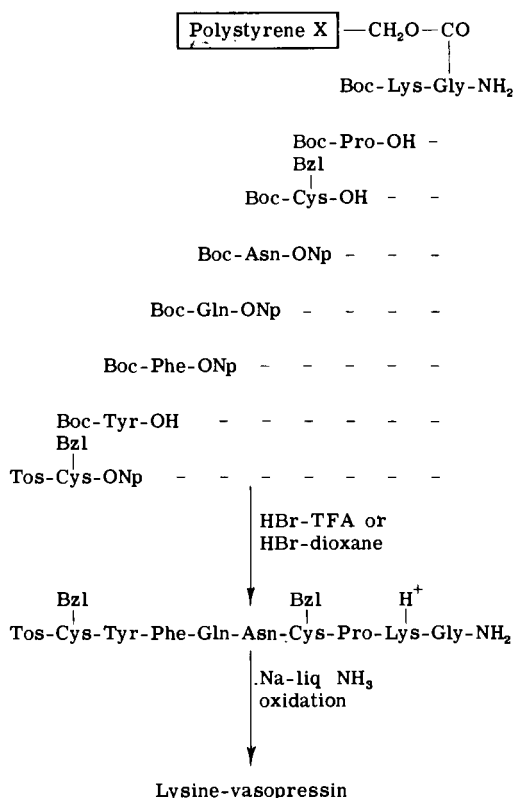
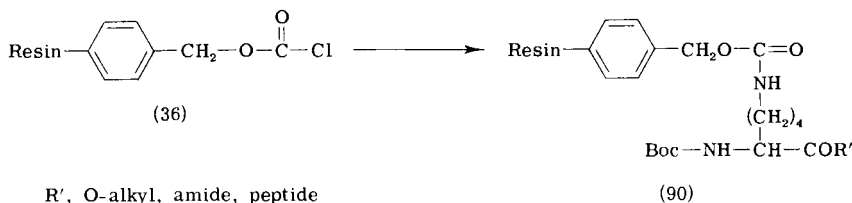


FIG. 7. A solid-phase synthesis of lysine-vasopressin with attachment of peptide to resin through the ϵ -amino group of the lysine residue (Meienhofer and Trzeciak, 1971).

chloroformoxymethyl resin. For example, reaction of the ϵ -amino group of a lysine residue with a chloroformoxymethylated resin gives a benzyloxycarbonyl-type bond (90). Peptide chain elongation can then be carried out in the proven direction from the C-terminal toward the N-terminal.* [8-Lysine]-vasopressin has been synthesized through such a side-chain attachment to the solid-phase (Meienhofer and Trzeciak, 1971) as shown in

* A solid-phase synthesis of a dipeptide in opposite direction (starting from the N-terminal amino acid) through linkage of the α -amino group to a chloroformoxymethylated resin (36) was explored (Letsinger and Kornet, 1963; Letsinger *et al.*, 1964). To prepare larger peptides, a "racemization-free" method would be required for peptide bond formation. The use of the azide method for this approach was studied recently by Felix and Merrifield (1970) and was judged inadequate because of marginal yields.

Fig. 7. [We have recently learned that side-chain attachment through the δ -amino group of ornithine to chloroformoxymethyl resin (36) had already been used in a synthesis of [Gly⁵, Gly¹⁰]-gramicidin S (Sklyarov and Shashkova, 1969).] For the attachment, a solution of Boc-Lys-Gly-NH₂ in dimethylformamide, containing triethylamine, was stirred with chloroformoxymethyl resin for 50 hours at room temperature resulting in a substitution rate of about 0.3 mmole/gm. Unreacted chlorocarbonyl groups were inactivated by treatment with ammonia in benzyl alcohol. For chain elongation the standard procedure (Marshall and Merrifield, 1965) was followed. Removal of the resin from the completed nonapeptide chain was achieved after treatments with (a) hydrogen bromide in dioxane or (b) hydrogen bromide in trifluoroacetic acid for 15 minutes; conditions which are somewhat milder or shorter, respectively, than those required for complete cleavage of the commonly used benzyl ester linkage. Still milder methods should be applicable with some of the above alternative linkages.



3. Quantitation of Resin-Bound Amino Acids

Determination of the exact amount of the first amino acid covalently bound to the solid-phase is by no means a simple task. In most investigations dealing with the standard benzyl ester attachment to cross-linked polystyrene resins, hydrolysis by refluxing with a 1:1 mixture of 12 *N* hydrochloric acid and dioxane for 18–48 hours followed by amino acid analysis has been used (Marshall and Merrifield, 1965; Stewart and Young, 1969). In this manner values considerably lower than the actual degree of substitution can sometimes be obtained, probably due to some decomposition and/or adsorption of amino acids during heating with polystyrene (Wieland *et al.*, 1969a). Independent determination of the amino acid content, e.g., by one of the titration methods, discussed below (Section IV,D,1), after cleavage of the N-protecting group are, therefore, highly recommended, especially since total yields are generally based on the amino acid content of the starting resin.

Alternative cleavage conditions have been proposed for more accurate determination of amino acid contents in resins. Grahl-Nielsen and Tritsch

(1969) recommend removal by HBr–trifluoroacetic acid in a specially designed analytic vessel, followed by ninhydrin color determination. Scotchler *et al.* (1970) obtained optimal amino acid values after hydrolysis with a deaerated mixture of 12 *N* HCl and propionic acid (1:1) at 130° for 2 hours. Base catalyzed alcoholysis followed by standard acid hydrolysis (6 *N* HCl, 110°, 15 hours) has also given acceptable values.

C. PROTECTION–DEPROTECTION

1. α -Amino Group Protection

High demands are placed on the choice of α -amino protecting groups because the repetitive solid-phase procedure requires highly selective deprotection. Three essential conditions must be met during deprotection: (a) quantitative removal of the $N\alpha$ -protecting group; (b) complete stability of the peptide to polymer bond; and (c) complete stability of certain side-chain protecting groups, in particular those of the ω -amino functions.

The initial use (Merrifield, 1962, 1963) of the most widely used amino protecting group in solution synthesis, the benzyloxycarbonyl group of Bergmann and Zervas (1932), required ring nitration or bromination of the resin to increase the stability of the peptide to polymer linkage. However, the deprotection conditions were altogether too severe for general use, especially for the synthesis of large peptides containing trifunctional amino acids.

a. The tert-Butyloxycarbonyl Group. The usefulness of the *tert*-butyloxycarbonyl group (Boc) (Anderson and McGregor, 1957; McKay and Albertson, 1957) for the solid-phase procedure was next investigated by Merrifield (1964a,b). Since then it has been used in the majority of solid-phase syntheses. Merrifield (1969a) cautions, however, that the $N\alpha$ -*tert*-butyloxycarbonyl protection “is probably not the final answer.”

Boc-Amino acids are readily obtained by well-described and controlled procedures using a variety of different reagents. The newer developments are briefly discussed later in this section.

Deprotection has been effected in solid-phase synthesis by treatment with 1 *N* HCl in glacial acetic acid (for 20–40 minutes), with 4 *N* HCl in dioxane (for 30 minutes) (Stewart and Woolley, 1965a; Merrifield, 1966), with anhydrous trifluoroacetic acid (TFA) (15 minutes to 2 hours) (Kusch, 1966a,b; Anfinsen *et al.*, 1967; Takashima *et al.*, 1968; Manning, 1968), or with freshly prepared 50% trifluoroacetic acid in methylene chloride (Gutte and Merrifield, 1969). Karlsson *et al.* (1970b) concluded from a comparison of three reagents, trifluoroacetic acid–methylene chloride, 1:1 (v/v), 3.8 *N* HCl–dioxane, and 1 *N* HCl–acetic acid, that freshly prepared

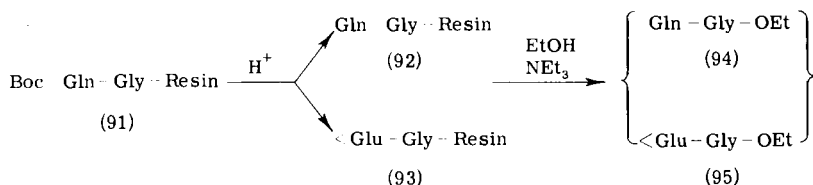
50% trifluoroacetic acid in methylene chloride produced the best results. It effected the fastest (10–30 minutes) and most reliable cleavage, and it was judged to be superior to 4 *N* HCl in dioxane. It was, however, noticed that cleavage of the benzyl ester linkage to the resin occurred to a small degree during each treatment with the TFA–CH₂Cl₂ reagent. A thorough study by F. H. C. Stewart (1967) showed that ring substitution with methyl groups greatly enhances the lability of benzyl esters toward trifluoroacetic acid. The polyethylene backbone of polystyrene should exert a similar influence. Considerable losses of peptide from resin have been reported by Merrifield and Marglin (1967) in a synthesis of insulin A-chain, by Gutte and Merrifield (1969, 1971) during their ribonuclease A preparation, by Bayer *et al.* (1968a) (ferredoxin preparation), and by Norris *et al.* (1971) in a model system. Therefore, a lower concentration (20% trifluoroacetic acid in methylene chloride) has recently been recommended (Gutte and Merrifield, 1971), which reportedly effects still rapid and complete Boc group cleavage (Krumdieck and Baugh, 1969).

Reagents which might be expected to affect benzyloxycarbonyl groups or benzyl esters much less than hydrogen chloride or trifluoroacetic acid are formic acid, boron trifluoride in acetic acid, or *p*-toluenesulfonic acid. Formic acid cleaved *tert*-butyloxycarbonyl groups effectively in solution synthesis leaving benzyl esters completely intact (Halpern and Nitecki, 1967); but it reacted too slowly with resin-linked Boc-aminoacyl residues to be generally useful (Karlsson *et al.*, 1970b). Boron trifluoride in glacial acetic acid exhibits very high selectivity in cleaving Boc groups rapidly and completely without affecting benzyloxycarbonyl groups in solution synthesis (Hiskey and Adams, 1966; Meienhofer, 1970; Schnabel *et al.*, 1971c); it has not yet been tested in solid-phase synthesis; neither has *p*-toluenesulfonic acid (see, however, Southard *et al.*, 1969, 1971c).

Problems have been encountered with glutamine residues. During a synthesis of deaminooxytocin (Takashima *et al.*, 1968), incomplete coupling was observed when attempting to extend the chain beyond the glutamine residue. Ring closure due to pyroglutamyl formation during deprotection with HCl in acetic acid (or other solvents) was assumed to occur as a side reaction. The problem was circumvented by deblocking Boc-glutamine residues with anhydrous trifluoroacetic acid or with 50% trifluoroacetic acid in methylene chloride (Blake *et al.*, 1970). HCl has since that time generally been avoided for the deprotection of glutamine residues. However, until very recently the alleged pyroglutamyl formation has never really been investigated. There has been no published report of any experimental proof for pyroglutamic acid (pyrrolidone carboxylic acid) formation during solid-phase synthesis except for a footnote [24] in Beyerman and In't Veld

(1969). An examination of literature frequently quoted (e.g. Smyth *et al.*, 1962) for alleged pyroglutamyl formation during Edman degradation with the use of HCl in acetic acid at 100°, revealed that the effect had only been discussed as “a possible impediment” in the original Edman procedure. Alternatively, incomplete reaction in a solid-phase synthesis cycle after *tert*-butyloxycarbonylglutamine incorporation could be due to incomplete cleavage of the N-protecting *tert*-butyloxycarbonyl group from the glutaminyl residue by 1 *N* HCl in acetic acid. To obtain complete and rapid deprotection, 3–4 *N* HCl in dioxane had to be used by Dorman *et al.* (1972).

Recently Beyerman *et al.* (1973b) investigated the problem using Boc-Gln-Gly-Resin (91) as a model system. Pyroglutamic acid (93), formed during acidolytic deprotection, was determined by thin-layer chromatography after removal of the solid support by base catalyzed ethanolysis and comparison of the product (94) + (95) with authentic synthetic <Glu-Gly-OEt (95). The results are summarized in Table VIII. 4 *N* HCl in dioxane caused no detectable pyroglutamyl formation and 1 *N* HCl in acetic acid caused little. Anhydrous trifluoroacetic acid produced 4% pyroglutamyl formation in the model system after 70 hours at 23°. Dilution of trifluoroacetic acid with methylene chloride gave striking increases in pyroglutamyl formation. Incubation of deprotected Gln-Gly-Resin (92) with acetic acid (as used in washing operations) produced the highest amount of pyroglutamyl peptide.*



To protect acid-sensitive tryptophan residues during cleavage of the *tert*-butyloxycarbonyl group, thiol-containing compounds have been added to the reagents. Marshall (1968) recommended the use of 1% β-mercaptoethanol in 1 *N* HCl–HOAc. Addition of β-mercaptoethanol to trifluoroacetic acid in methylene chloride has also given good results (Wang and Merrifield, 1969b; Eylar *et al.*, 1970). However, 0.08 *M* dithiothreitol added to 50% trifluoroacetic acid in methylene chloride (Li and Yamashiro, 1970) would seem to be a less malodorous reagent. Substitution of the indole moiety occurs when the *o*-nitrophenylsulfenyl group is cleaved by

* These conditions are similar, with respect to pH, to those under which Smyth *et al.* (1963) observed pyroglutamyl formation in peptides with N-terminal glutamine residues during column chromatography of enzymic digests.

Table VIII—Formation of Pyroglutamyl-Peptide during Deprotection of Boc-Gln-Gly-Resin ^a

Acid solvents (at 23°)	% Formation at different times		
	½ Hour	1 Hour	70 Hours
4 N HCl-Dioxane	—	—	—
1 N HCl-HOAc	—	—	1
TFA	—	—	4
TFA-CH ₂ Cl ₂ (1:1 v/v)	—	—	11
TFA-CH ₂ Cl ₂ (1:5 v/v)	—	Trace	17
HOAc-H ₂ O (4:1 v/v) ^b	—	—	45
HOAc ^b	1-2	2-3	60

^a Estimated after ethanolysis, by comparison on tlc with equivalent amounts of Glu-Gly-OEt. From Beyerman *et al.*, (1973).

^b After previous removal of the Boc-group with TFA, 15 minutes at 23°.

HCl-HOAc in tryptophan containing peptides (Wünsch *et al.*, 1967; Scoffone *et al.*, 1968).

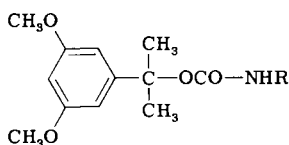
For several years it had been tacitly assumed that cleavage of the *tert*-butoxycarbonyl group proceeded always to completion, but in 1968 Nitecki [*First American Peptide Symposium*, New Haven, August, 1968 (see Nitecki and Goodman, 1970)] pointed out that this might not always be the case. The danger of incomplete deprotection giving rise to the generation of failure sequences or truncated sequences (see Section II,C), has been the subject of a communication by Chou *et al.* (1971). Incomplete cleavage of N α -Boc groups even after repeated treatment with HCl-HOAc (twice for 30 minutes) was observed by Jorgensen *et al.* (1970a) and by Dorman and Markley (1971). To optimize deprotection, twice repeated treatment with 50% TFA-CH₂Cl₂ reagent has been routinely adopted by some groups of workers (Manning *et al.*, 1970; Hancock *et al.*, 1971; Marshall *et al.*, 1973; Shapira *et al.*, 1971). During syntheses of melanotropin sequences, Chou *et al.* (1971) observed incomplete deprotection. In efforts to obtain complete N α -Boc removal, several reagents and mixtures were studied including 5.6 N HCl-dioxane, 5.4 N HCl in dimethylsulfoxide-methylene chloride (1:1), and pretreatment of peptide-resin with 1.5 M urea in dimethylformamide followed by acid. However, the observations of Chou *et al.* (1971) about incomplete deprotection by trifluoroacetic acid-methylene chloride (1:1) could not be confirmed (Li, 1971).

Blake and Li (1968) pointed out that the copolystyrene-2% divinylbenzene resin retains dioxane from washing in such amounts so that 5.8 N HCl in dioxane should be used to obtain an HCl normality of about 3.5-4 in the dioxane resin mixture during the deprotection step. Gutte and Merrifield

(1971) overcame similar dilution effects by introducing a 2-minute preliminary wash with 50% TFA-CH₂Cl₂ before the actual deprotection step.

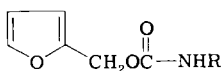
b. Other N α -Protecting Groups. Investigations of the applicability in solid-phase synthesis of the *tert*-amyloxycarbonyl group (Inukai *et al.*, 1968), the *o*-nitrophenylsulfenyl group (Kessler and Iselin, 1966; Najjar and Merrifield, 1966; Fridkin *et al.*, 1968; Marshall, 1968), *p*-methoxybenzyloxycarbonyl group (Weygand and Ragnarsson, 1966), and 3,5-dimethoxybenzyloxycarbonyl group (Dimoz) Wieland and Birr, 1967b) have been reviewed by Merrifield (1969a, Table III).

A further development of the Dimoz group is the highly acid labile α,α -dimethyl-3,5-dimethoxybenzyloxycarbonyl protecting group (96) (Birr *et al.*, 1973). Its usefulness has been demonstrated through a solid-phase



(96)

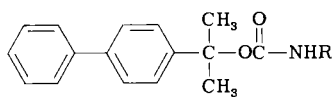
synthesis of antamanide, using 5% trifluoroacetic acid in anhydrous methylene chloride for deprotection (15 minutes at 20°). Alternative N α -protecting groups that have been studied include the furfuryloxycarbonyl group (97) (Foc) (Losse and Neubert, 1968a, 1970b), and the triphenylmethyl group (Losse and Neubert, 1968a).



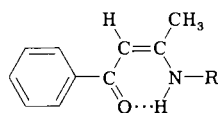
(97)

Comparing the relative merits of the N α -Boc, N α -Foc, N α -Tri, and N α -Nps groups in syntheses of a model tetrapeptide, the Boc group was found to be the most useful, the Nps group the least useful (Losse and Neubert, 1968a). Visser and Kerling (1970), however, took advantage of the mild conditions [0.1 N HCl in acetic acid-chloroform (Najjar and Merrifield, 1966)] for cleavage of the *o*-nitrophenylsulfenyl group from the Nps-Glu(OBu^t)-Cit-Gln-His(Bzl)-Leu-resin without affecting the γ -*tert*-butyl ester group. Sklyarov and Shashkova (1969) used N α -Nps protection over five cycles in a synthesis of gramicidin S.

Very promising among recently developed amine protecting groups are both the 2-(*p*-biphenyl)isopropylloxycarbonyl group (98) (Sieber and Iselin, 1968a,b) and the 2-benzoyl-1-methylvinyl group (99) (Southard *et al.*, 1969). The Bpoc group has already proven its value in several syntheses



(98)

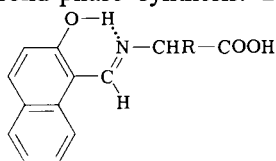


(99)

(Wang and Merrifield, 1969a,b, 1971; Yamashiro *et al.*, 1972). Its cleavage is effected under very mild conditions using 0.5% trifluoroacetic acid in methylene chloride (10 minutes), 0.05 *N* HCl in methylene chloride (few minutes), or 0.04 *N* HCl in chloroform (Blake and Li, 1971). Under these conditions the peptide to resin benzyl ester linkage is completely stable over prolonged periods of time (>100 hr). The 2-benzoyl-1-methylvinyl amine protecting group was first used for peptide synthesis by Dane *et al.* (1962) and has been applied to the solid-phase procedure by Southard and collaborators (1969). Cleavage of this group is achieved with 0.4 *N* HCl in tetrahydrofuran (30 minutes) or 0.4 *N* *p*-toluenesulfonic acid in tetrahydrofuran (Southard *et al.*, 1969, 1971c). These conditions were mild enough to use a benzhydryl ester bond for peptide to resin attachment. The efficiency of the system was shown by syntheses of Leu-Ala-Gly-Val and glucagon-(18-29)-dodecapeptide.

Ragnarsson *et al.* (1970a) studied thoroughly the behavior of the benzyloxycarbonyl, *tert*-butyloxycarbonyl, *o*-nitrophenylsulenyl, *p*-methoxybenzyloxycarbonyl, and 2-(*p*-biphenyl) isopropylloxycarbonyl groups toward ten deprotecting reagents. The best system was judged to be α -amino protection by the Bpoc group and its removal by 0.1 *M* trifluoroacetic acid in methylene chloride. The theoretical free amine value was obtained rapidly (5–10 minutes), and the benzyl ester linkage of peptide to resin was hardly affected at all.

Khosla *et al.* (1972) described the use of *N* α -(2-hydroxy-1-naphthal) amino acids (100) for solid-phase synthesis. Deprotection was effected



(100)

either by 0.5 *N* aqueous HCl in dioxane or by 7% benzylamine in methylene chloride. Esko *et al.* (1968) previously used this system as an analytic procedure for resin-amine determination (see Section IV,D,2).

2. Side-Chain Protection

The requirements for useful combinations of protecting groups are much more stringent in solid-phase synthesis than in solution synthesis. Cleavage

of the α -amino protection must always be 100% complete during the course of chain elongation, while absolutely no loss of side-chain protection should occur. The combination of protection based on the benzyl group (e.g., benzyl ester, benzyloxycarbonyl group) with that based on *tert*-alkyl groups (e.g., *tert*-butyl ester, *tert*-butyloxycarbonyl group) has proven to be extremely valuable in solution synthesis (Schröder and Lübke, 1966). However, in solid-phase synthesis this combination was soon found to be inadequate. For example, Yaron and Schlossman (1968) selected *N*-benzyloxycarbonyl and *N* α -*tert*-butyloxycarbonyl protection of lysine for a synthesis of nonalysine. The Boc group was cleaved in each cycle with 1 *N* HCl in acetic acid. At the end of the synthesis column chromatographic fractionation of the crude product revealed that the yield of the desired nonalysine was only 35%, while the yield of peptides of shorter chain lengths was 22%. Peptides containing ten to seventeen residues of lysine were obtained in 43% yield (Fig. 8). The higher oligomers apparently arose from the growth of peptide chains at ϵ -amino groups (Fig. 9) as a consequence of small losses of benzyloxycarbonyl groups in each deprotection step.

Small amounts of cleavage of the benzyloxycarbonyl group from *N*-benzyloxycarbonyllysine residues were also observed by Grahl-Nielsen and Tritsch (1969). Ontjes and Anfinsen (1970) have measured the extent of cleavage of *N*-benzyloxycarbonyl groups by various acid reagents in solution and in solid phase (Table IX). For instance, 60% free lysine was obtained from Boc-Lys(Z) by a 24-hour treatment in an anhydrous trifluoroacetic acid solution. Ragnarsson *et al.* (1970a) observed about 2%

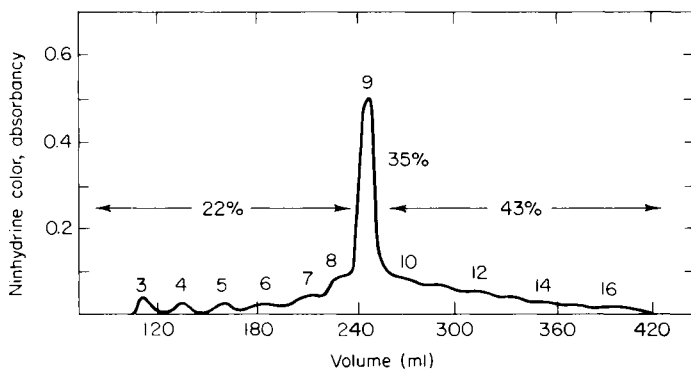


FIG. 8. Chromatography of crude *N* α -Dnp-nonallysine (2 mg) prepared by the solid-phase method. *N* α -Boc-*N* ϵ -Z-lysine was used. The Boc groups were cleaved with 1 *N* HCl in acetic acid. A CM-cellulose column (1.3 $\times$ 40cm) in Na⁺ form, equilibrated with 0.1 *M* NaCl was used with a constant volume (300 ml) gradient from 0.2–1.0 *M* NaCl at a 30 ml/hour flow rate. Numbers indicate lysine residues in products. The components containing over nine lysine residues originated from side-chain branching. From Yaron and Schlossman (1968).

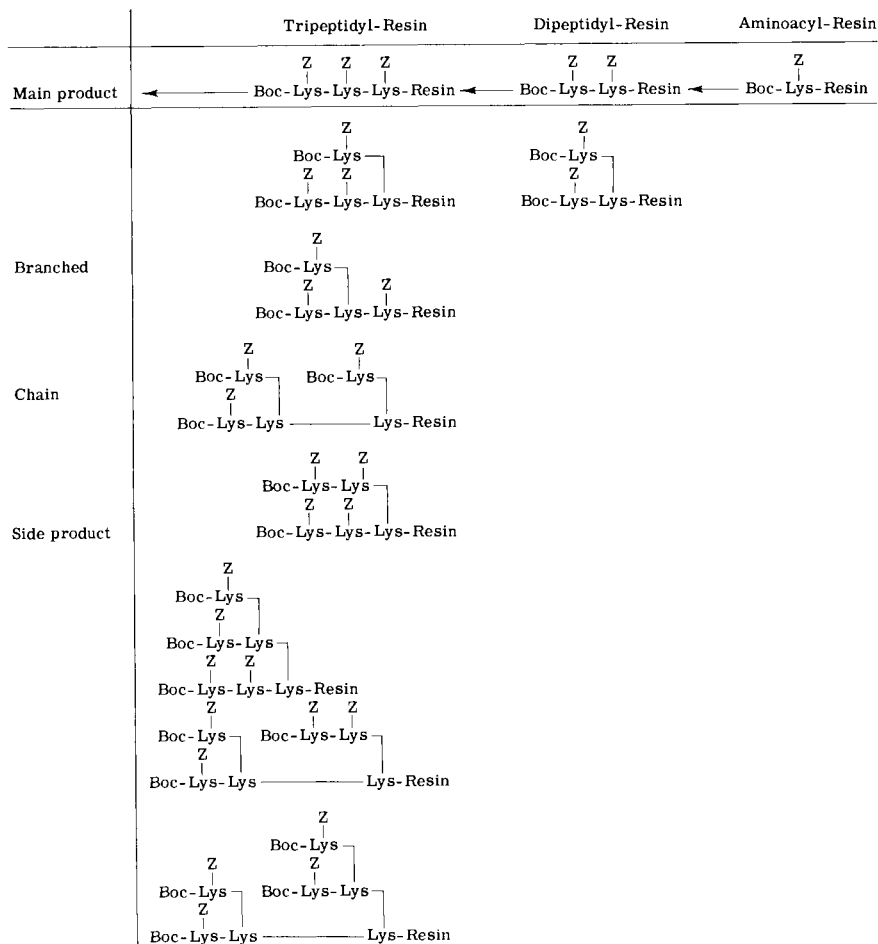


FIG. 9. Formation of branched-chain peptides as a consequence of partial loss of protecting groups from side-chain ω -amino-functions during the deprotection of the α -amino function.

benzyloxycarbonyl group cleavage after treating Z-Ile-Ala-Resin for 18 hours with 50% trifluoroacetic acid in methylene chloride.

Deficiencies of this combination of protecting groups were also demonstrated by observations of partial cleavage of benzyl ester protecting groups (used, e.g., for glutamic or aspartic acid side-chain functions) during solid-phase synthesis (Nitecki and Goodman, 1970; Karlsson *et al.*, 1970b). These types of side reactions present a serious problem in solid-phase syn-

Table IX—Stability of ϵ -Benzyloxycarbonyl Blocking Group to Acid Reagents ^a

Lysine derivative	Reagent	Time	Result
α -Boc- ϵ -Z-lysine	4N HCl-dioxane	15 minutes	2% Free lysine
		1 hour	7% Free lysine
		24 hours	20% Free lysine
α -Boc- ϵ -Z-lysine	1N HCl-acetic acid	24 hours	39% Free lysine
α -Boc- ϵ -Z-lysine	Trifluoroacetic acid	24 hours	Over 60% free lysine
α -Boc- ϵ -Z-lysine resin	4N HCl-dioxane (cleavage by ammonolysis)	15 minutes	Major product ϵ -Z-lysine amide, trace lysine amide
		24 hours	Major products lysine amide and free lysine

^a From Ontjes and Anfinson, 1970. Lysine was measured by quantitative amino acid analysis. ϵ -Z-lysine amide and lysine amide were estimated by thin layer chromatography (butanol 4; water 2; pyridine 1; acetic acid 1). α -Boc- ϵ -Tfa-lysine was exposed to 4 N HCl-dioxane for 24 hours with formation of less than 2% free lysine by amino acid analysis.

thesis because all side-products are retained throughout the synthesis (see Fig. 9). With increasing chain length the large number of accumulated and closely related contaminating side-products will form or contribute to formation of microheterogeneous mixtures similar to those arising from failure sequence accumulation. Assuming, for example, side-product formation at a rate of 1% per peptide bond, the accumulated impurities would amount to about 40% in solid-phase preparations containing fifty amino acid residues.

To prevent the occurrence of undesired fission of side-chain protecting groups, in particular those of the ω -amino functions during the deprotection of the α -amino function, a ratio of stability toward the deprotecting reagent of at least 1:1000, but preferably higher is required (Rudinger and Gut, 1967; Sieber and Iselin, 1968a). The use of substituted benzyloxycarbonyl groups with increased stability toward acidolytic deprotecting reagents for the $N\alpha$ -Boc group provides one possible solution. Thus, *p*- and *m*-chlorobenzyloxycarbonyl protection of the lysine ϵ -amino group has been employed by Noda *et al.* (1970a) for solid-phase syntheses. *p*-Nitrobenzyloxycarbonyl side-chain protection of ornithine was used in combination with *p*-methoxybenzyloxycarbonyl protection of the α -amino function in an improved synthesis of gramicidin S (Ohno *et al.*, 1971). The diisopropylmethoxycarbonyl group (Sakakibara *et al.*, 1970) exhibits an even greater stability towards HCl in acetic acid than the *p*-chlorobenzyloxycarbonyl group [and it is completely cleaved by HF during the final removal of all protecting groups from the completed peptide].

Another possible solution would be a milder deprotecting agent for the

cleavage of *N* α -*tert*-butyloxycarbonyl groups which would leave the *N* ϵ -benzyloxycarbonyl groups fully intact. Baugh *et al.* (1970b) employed 20% trifluoroacetic acid in methylene chloride; and the possible use of 10% solutions has been investigated (Ragnarsson *et al.*, 1970a), but Boc cleavage becomes too slow to make the latter system generally useful. A sevenfold molar excess of mercaptoethanesulfonic acid (20% solution in glacial acetic acid) cleaves Boc groups quantitatively in 30 minutes at room temperature without affecting benzyloxycarbonyl or benzyl ester groups (Loffet and Dremier, 1971).

Combinations of protecting groups that have so far been used are compiled for individual amino acids (or groups of homologous amino acids) in Table X. Literature quoted in this table is only a small selection of all published applications; a more extensive listing of literature up to early 1969 has been compiled by Losse and Neubert (1970a). In the overwhelming majority of syntheses the *tert*-butyloxycarbonyl group has served as *N* α protection. It is also evident from the table that for the great majority of cases side-chain protection is based on the benzyl group in spite of the shortcomings discussed above. Not many alternative groups have so far been examined except for histidine, arginine, and cysteine.

Histidine has been a problematic amino acid for solid-phase synthesis. *N*^{im} unprotected Boc-histidine has been used (e.g. Loffet, 1967; Gutte and Merrifield, 1969, 1971; Flouret, 1970), but many authors warn of (a) incomplete reaction (Chillemi and Merrifield, 1969; Ontjes and Anfinson, 1970; Yajima *et al.*, 1970b; Stewart *et al.*, 1972; Yamashiro *et al.*, 1972; Schaich and Schneider, 1972; Beyerman *et al.*, 1971b), (b) very low solubility and consequently adsorption to the solid phase causing great difficulties in removing excess reactant by washing (Schaich and Schneider, 1973), and (c) condensation of Boc-amino acids to the imidazole moiety in subsequent cycles (Yamashiro *et al.*, 1972). The benzyl group for imidazole protection provides improved coupling, but Boc-His(Bzl) is still of low solubility. Furthermore, final removal of the *N*^{im}-benzyl protection by sodium in liquid ammonia causes side reactions, and removal by catalytic hydrogenation is slow or sometimes fails. *N*^{im}-Benzyloxycarbonyl protection has been used (Ontjes and Anfinson, 1969a), but the limited stability of this group restricts its usefulness. Both the *N*^{im}-2,4-dinitrophenyl and the *N*^{im}-*p*-toluenesulfonyl protection have provided satisfactory results. The *N*^{im}-Dnp group is completely stable under standard solid-phase procedures but easily removed by thiolysis (Shaltiel, 1967; Chillemi and Merrifield, 1969; Marshall *et al.*, 1970; Shaltiel and Fridkin, 1970; Stewart *et al.*, 1972). The *N*^{im}-tosyl group is completely cleaved by hydrogen fluoride during removal of the solid support from the final product (Fujii and Sakakibara, 1970). Hydrogen chlo-

Table X—Side-Chain Protection Used in Solid-Phase Synthesis in Combination with *N* α -*tert*-Butyloxycarbonyl Protection

Amino acid	Side-chain protecting group	Selected literature examples
Arginine	Nitro (NO ₂) Stabile: HCl, TFA, HBr-TFA Cleaved: H ₂ -Pd, HF	Merrifield (1964b,c, 1966); Stewart <i>et al.</i> (1966); Schöllmann (1967); Young <i>et al.</i> (1967, 1968b); Lenard and Robinson (1967); Thampi <i>et al.</i> (1968); Gutte and Merrifield (1969, 1971)
	<i>p</i> -Toluenesulfonyl (Tos) Stabile: HCl, TFA, HBr-TFA Cleaved: Na-liq. NH ₃ , HF	Marglin and Merrifield (1966); Merrifield and Marglin (1967); Blake and Li (1968); Weber and Weitzel (1968); Meienhofer <i>et al.</i> (1970b)
	Protonation (H ⁺)	Loffet (1967)
Aspartic, glutamic acid	Benzyl ester (OBzl) Stabile: HCl ^a , TFA ^a Cleaved: H ₂ -Pd	Marshall and Merrifield (1965); Stewart <i>et al.</i> (1966); Marglin and Merrifield (1966); Merrifield and Corigliano (1968); Nitecki and Goodman (1970); Gutte and Merrifield (1969, 1971)
	<i>tert</i> -Butyl ester (OBu ^t) ^b	Visser <i>et al.</i> (1968); Shchukina <i>et al.</i> (1968)
Cysteine	Benzyl thioether (Bzl) Stabile: HCl, TFA, HBr-TFA, HF ^a Cleaved: Na-liq. NH ₃ , HF ^a	Marglin and Merrifield (1966); Zahn <i>et al.</i> (1967a,b); Hörnle (1967); Bayer and Hagenmaier (1968); Takashima <i>et al.</i> (1968); Meienhofer and Sano (1968); Gutte and Merrifield (1969, 1971)
	S-Acetyl (Ac); S-benzoyl (Bz); S-benzyl (Bzl); S-benzyloxycarbonyl (Z); S-benzylthio-methyl (Btm); S-di-phenylmethyl (Dpm); S-ethoxycarbamoyl (Ec); S- <i>p</i> -methoxy-benzyl (Meb); S-tetra-hydropyranyl (Thp); S-triphenylmethyl (Tri)	Hammerström <i>et al.</i> (1970)
	Disulfide Stabile: HCl, TFA Cleaved: R-SH, Na ₂ SO ₃	Lunkenheimer and Zahn (1970a,b)
	S-Et, S-alkyl Stabile: HCl Cleaved: R-SH, Na ₂ SO ₃	Inukai <i>et al.</i> (1968); Weber (1969); Weber <i>et al.</i> (1969, 1971)

Table X, *cont.*

Amino acid	Side-chain protecting group	Selected literature examples
Histidine	Benzyl (Bzl) Stabile: HCl, TFA, HBr-TFA, HF Cleaved: H ₂ -Pd, Na-liq. NH ₃	Marshall and Merrifield (1965); Blake and Li (1968); Weber and Weitzel (1968)
	<i>p</i> -Toluenesulfonyl (Tos) ^a Stabile: HCl, TFA Cleaved: HF	Fujii and Sakakibara (1970)
	Benzyloxycarbonyl (Z) Stabile: HCl, TFA Cleaved: H ₂ -Pd	Ontjes and Anfinen (1969a,b)
	2,4-Dinitrophenyl (Dnp) Stabile: HCl, TFA, HBr-TFA Cleaved: R-SH	Chillemi and Merrifield (1969); Chillemi (1971); Stewart <i>et al.</i> (1972)
	<i>tert</i> -Butyloxycarbonyl (Boc) ^a Stabile: 0.05-0.01 N, HCl in CHCl ₃ Cleaved: TFA, HBr-TFA, HF	Yamashiro <i>et al.</i> (1972); Blake <i>et al.</i> (1972); Schaich and Schneider (1973)
Lysine	Benzyloxycarbonyl (Z)	Merrifield (1964c); Yaron and Schlossman (1966); Marglin and Merrifield (1966); Zahn <i>et al.</i> (1967a,b); Lenard and Robinson (1967); Grahl-Nielsen and Tritsch (1969); Gutte and Merrifield (1969, 1971)
Ornithine	Stabile: HCl ^a , TFA ^a Cleaved: HBr-TFA, H ₂ -Pd, HF	
	<i>p</i> - or <i>m</i> -Chlorobenzyl-oxycarbonyl	Noda <i>et al.</i> (1970a)
	<i>p</i> -Toluenesulfonyl (Tos) Stabile: HCl, TFA, HBr-TFA, HF Cleaved: Na-liq. NH ₃	Stewart and Woolley (1966); Meienhofer and Sano (1968); Klostermeyer (1968)
	Trifluoroacetyl (Tfa) Stabile: HCl, TFA, HBr-TFA, HF Cleaved: 1 M piperidine	Anfinen <i>et al.</i> (1967); Ontjes and Anfinen (1969a,b)
Serine	Benzyl ether (Bzl)	Merrifield (1964a,b,c); Marshall and Merrifield (1965); Lenard and Robinson (1967); Manning <i>et al.</i> (1968a); Gutte and Merrifield (1969, 1971)
Threonine	Stabile: HCl, TFA Cleaved: HBr-TFA, HF	

Table X, *cont.*

Amino acid	Side-chain protecting group	Selected literature examples
Tyrosine	2,2,2-Trifluoro-1-benzyl-oxycarbonylaminoethyl (Ztf) Stabile: HCl-HOAc Cleaved: HBr-TFA	Polzhofer (1969b)
	Benzyl ether (Bzl) Stabile: HCl, TFA Cleaved: HBr-TFA, HF	Marshall and Merrifield (1965); Park <i>et al.</i> (1967); Weber <i>et al.</i> (1967); Manning (1968); Bayer <i>et al.</i> (1968a,b)
	Benzyloxycarbonyl (Z) Stabile: HCl, TFA Cleaved: HBr-TFA, HF	Semkin <i>et al.</i> (1968)

^a Incomplete stability.

^b Used in combination with *N* α -*o*-nitrophenylsulfenyl protection.

^c Incomplete cleavage.

^d Used in combination with *N* α -*tert*-amyloxycarbonyl protection.

^e Use of Bpoc-His(Boc) by Yamashiro *et al.* (1972) and Blake *et al.* (1972); use of Boc-His(Boc) by Schaich and Schneider (1973).

ride in organic solvents must not be used for α -amino deprotection in syntheses employing *N*^{im}-tosylhistidine, since the tosyl group is cleaved by anhydrous HCl. The *N*^{im}-*tert*-butyloxycarbonyl group is the most recent development in histidine side-chain protection. Preparations of *N* α -2-(*p*-biphenyl) isopropylloxycarbonyl-*N*^{im}-*tert*-butyloxycarbonylhistidine (Yamashiro *et al.*, 1972), and of *N* α ,*N*^{im}-bis(*tert*-butyloxycarbonyl) histidine (Schnabel *et al.*, 1968a; Yamashiro *et al.*, 1972; Schaich and Schneider, 1973) have been reported. Several solid-phase syntheses of a heptapeptide [human growth hormone-(17-23)] using these derivatives have produced very good results (Yamashiro *et al.*, 1972). The stabilities of various imidazole protecting groups toward several reagents are compiled in Table XI.

Arginine is the only trifunctional amino acid for which more than one type of side-chain protection has been used over a longer period of time in solid-phase synthesis (see Table X). The nitro group has been used most frequently. Recently, Yamashiro *et al.* (1972) warned of side reactions leading to ornithine formation. These might occur during the final removal of the solid support by either hydrogen fluoride or hydrogen bromide in trifluoroacetic acid. No difficulty was observed when the *p*-toluenesulfonyl group was used for the protection of the guanido function. The tosyl group is readily cleaved by hydrogen fluoride (Mazur and Plume, 1968); alterna-

Table XI—Stability of Imidazole Protecting Groups ^a

Protecting group	6 N HCl, 110°	HCl-dioxane, 25°	TFA-CHCl ₃ , 25°	HF	NH ₃ -DMF, 25°	H ₂ -Pd	Na-liq. NH ₃	R-SH
Benzyl	—	—	—	—	—	+	+	—
Benzoyloxycarbonyl	+	—	—	+	+	+	+	—
<i>tert</i> -Butyloxycarbonyl	+	+	+	+	—	—	—	—
2,4-Dinitrophenyl	—	—	—	—	+	×	×	+
<i>p</i> -Toluenesulfonyl	+	+	—	+	+	+	+	—

^a Data in part from Stewart *et al.*, 1972. Explanation of symbols: —, stable; +, removable; ×, incompatible.

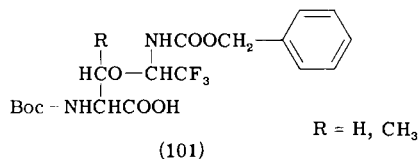
tively, sodium in liquid ammonia has been used after removal of the solid support.

The benzyl group was used almost exclusively until 1969 for the protection of the cysteine thiol function. Since treatment with sodium in liquid ammonia is the only reliable deprotection procedure for the *S*-benzyl group and since this is known to cause side reactions such as proline peptide bond cleavage, other groups have been investigated, see Table X. Several alkyl-mercapto groups (Inukai *et al.*, 1968; Weber, 1969; Weber *et al.*, 1969, 1971) provided effective thiol protection in solid-phase syntheses of oxytocin and insulin chain analogs.

The hydroxyl group of serine mostly has been protected as the benzyl ether (see Table X) to prevent carbodiimide mediated acylation. Garner *et al.* (1971) hoped that with active ester condensations (using, e.g., 2,4,5-trichlorophenyl esters) O-unprotected serine could be employed. Even then, however, peptide couplings could not be completed without some acylation of the hydroxyl by the active esters. This seemed not to have been noticed by Yajima *et al.* (1970b) who did use Boc-serine pentachlorophenyl ester.

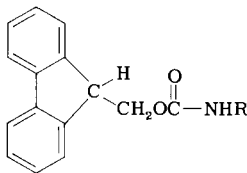
Threonine might be used without hydroxyl protection since its methyl group provides some steric shielding against undesired O-acylation. Thus, Loffet *et al.* (1971) did not observe any undesired O-acylation, however, the threonine residue was positioned in the peptide chain between a valine and a leucine residue. During a solid-phase synthesis of a [Thr⁶]-bradykinin analog, the threonine residue is in a less hindered environment and O-acylation was observed (Stewart *et al.*, 1967). It would, therefore, seem to be advisable to protect hydroxyl functions in solid-phase syntheses.

An alternative to the benzyl ether protection was examined by Polzhofer (1969b). He used the *O*-2,2,2-trifluoro-1-benzyloxycarbonylaminoethyl (Ztf) derivatives (101) of serine and threonine (Weygand *et al.*, 1968a) for solid-phase syntheses of model peptides. The Ztf groups were stable throughout the syntheses (1 *N* HCl-HOAc was used for deprotection) and were removed during the final hydrogen bromide in trifluoroacetic acid treatment.



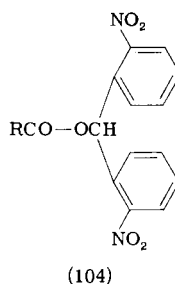
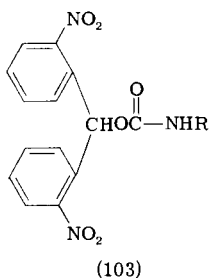
Side reactions connected with side-chain protecting groups were found to occur with aspartic acid and arginine residues. Transesterification of resin-bound β -benzyl aspartate to give the β -methyl ester was observed (Southard, 1971) when a triethylamine treatment was shortly followed, or preceded, by a methanol wash. Aspartyl β -alkyl ester residues, especially when next to glycine or serine residues can undergo cyclic diacylimide formation (Ondetti *et al.*, 1968a); see Section III,E,1 for more details. Ornithine formation from nitroarginine residues has been mentioned above. It would be misleading to conclude from the small number of reported side reactions that these do not occur at all in solid-phase synthesis, or less frequently than in solution synthesis. The reason for the relatively infrequent observation of side reactions is, of course, the inaccessibility of the growing peptide in the solid phase to analytic scrutiny when side reactions occur. Afterwards, side products are difficult to detect in the final product among other accumulated contaminants.

In conclusion, it would be desirable if alternative combinations of α -amino group and side-chain protection would be investigated. The potentially most promising system to-date, for which some experience has been gained already (Wang and Merrifield, 1969b; Yamashiro *et al.*, 1972) appears to be the Bpoc group for the α -amino protection and the *tert*-alkyl or the benzyl-based groups for side-chain protection. An interesting acid stable amine protecting group, the 9-fluorenylmethoxycarbonyl group (102), has been



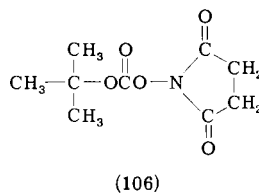
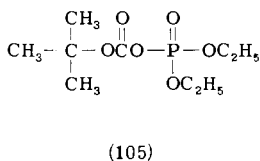
(102)

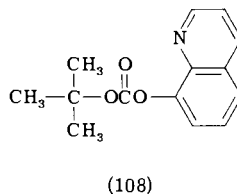
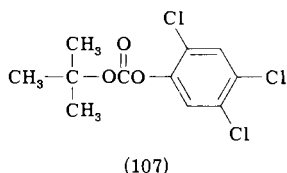
developed by Carpino and Han (1970). It can be removed by very mild base treatment. Other potentially useful protecting groups for solid-phase synthesis, in combination with some of the presently used groups, appear to be the photosensitive groups containing the 2,2'-dinitrodiphenylmethoxy moiety (103, 104) that have been described by Patchornik *et al.* (1970).



3. Preparation of Some Protected Amino Acids.

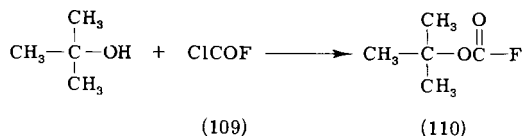
a. N α -tert-Butyloxycarbonylamino acid derivatives. The preparation of *N α -tert-butyloxycarbonylamino acids* (Anderson and McGregor, 1957; Schwyzer *et al.*, 1959) has been facilitated and improved by pH-stat controlled addition of base during the Schotten-Baumann acylation (Schnabel, 1967). Experimental aspects concerning the use of the reagent *tert*-butyloxycarbonyl azide (*tert*-butyl azidoformate) (Carpino, 1957) are discussed by Stewart and Young (1969). Boc-amino acids are obtained more readily (Yamashiro *et al.*, 1972) when the amount of solvents (dioxane-water, 1:1) is reduced to about one-third of that described by Schnabel (1967). Improved procedures for the preparation of the reagent have been described (Ovchinnikov *et al.*, 1965; Yajima and Kawatani, 1968; Sakai and Anselme, 1971; a warning concerning dangers of explosion has been published, see Yajima *et al.*, 1970a) and modified reagents based on the mixed anhydride or active ester methods have been proposed, such as *tert*-butylcarboxylic diethylphosphoric anhydride (105) (Tarbell and Insalaco, 1967), *tert*-butyl succinimidoyl carbonate (106) (Frankel *et al.*, 1966; Gross and Bilk, 1967, 1969), *tert*-butyl 2,4,5-trichlorophenyl carbonate (107) (Broadbent *et al.*, 1967), and *tert*-butyl quinolyl-8 carbonate (108) (Rzeszotarska and Wiejak, 1968a,b,c) to name a few.





tert-Butyloxycarbonyl chloride is not stable enough for storage (Choppin and Rogers, 1948); however, its use *in situ* for the preparation of *tert*-butyloxycarbonylamino acids has been described (Sakakibara *et al.*, 1969). Improved conditions for preparations of Boc-Asp(OBzl)-OH, Boc-Glu(OBzl)-OH (Polzhofer, 1969a), and of the dicyclohexylammonium salts of Boc-Ser(Bzl)-OH, Boc-Asp(OBu^t)-OH, Boc-Glu(OBu^t)-OH, and Boc-Glu(OBzl)-OH (Medzihradszky *et al.*, 1967) have been elaborated. An apparatus for the mechanized preparation of Boc-amino acids has been described (Birr and Frodl, 1970).

A very significant improvement was made with the development of the reasonably stable reagent *tert*-butyloxycarbonyl fluoride (110) (Schnabel *et al.*, 1968a,b,c) which is obtained from a reaction of carbonyl chloride fluoride* (109) with *tert*-butanol. Reaction conditions have been optimized



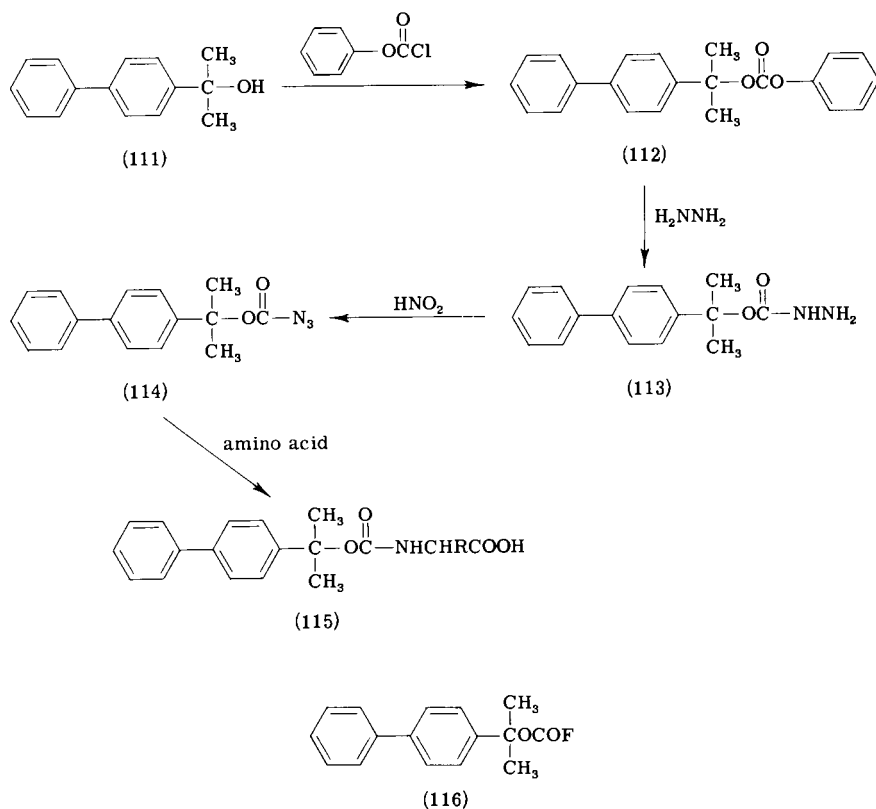
recently for the preparation of *N*-*tert*-butyloxycarbonyl derivatives of tri-functional amino acids (Schnabel *et al.*, 1971a), e.g., Boc-Asn-OH, Boc-Asn-ONp, Boc-Gln-OH, Boc-Cys(Boc)-OH, Boc-Cys(Boc)-OSu, Boc-His(Boc)-OH, Boc-His-OH, Boc-Ser-OH, Boc-Ser(Boc)-OH, Boc-Thr-OH, and Boc-Thr(Boc)-OH.

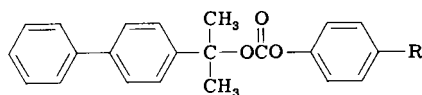
A list of protected amino acids which have been used in solid-phase peptide syntheses is to be found in the review of Merrifield (1969a). Attention has been drawn to the importance of using highly purified amino acid derivatives for solid-phase synthesis, and physical data (melting point, optical rotation) of repeatedly recrystallized Boc-amino acid derivatives have been compiled (Bayer *et al.*, 1968a) to aid in the assessment of purity of such compounds, particularly when they are of commercial origin.

*b. N*α-2-(*p*-Biphenyl)isopropylloxycarbonylamino Acid Derivatives.

* Tanks of carbonyl chloride fluoride can be obtained from Ozark Mahoning Co., 1870 South Boulder, Tulsa, Oklahoma 74119.

This protecting group (115), which was designed by Sieber and Iselin (1968a) especially for the solid-phase method, is introduced into amino acids with the use of the azide (114) (Sieber and Iselin, 1968b). This reagent was obtained from 2-(*p*-biphenyl)isopropanol via the carbonate (112) and the carbazate (113). The preparation of a large number of Bpoc-amino acid derivatives, including several with side-chain benzyl protection, by the use of azide (114) has been described by Wang and Merrifield (1969b). Schnabel *et al.* (1971b) prepared Bpoc-fluoride (116) which proved to be a most useful reagent allowing reactions in aqueous-organic solvent mixtures at temperatures between 0° and -10° to be completed within a few hours. Several 2-(*p*-biphenyl)isopropyl phenyl carbonates with different substituents in the phenyl ring (117) were also prepared and found to be thermally quite stable reagents giving good yields of Bpoc-amino acids in anhydrous solvents. Bpoc-amino acid derivatives are listed in Table XII.





(117)

R = $-\text{COOCH}_3$; $-\text{COCH}_3$; $-\text{C}_6\text{H}_5$ **Table XII**—Properties of *N* α -2-(*p*-Biphenyl)isopropoxycarbonylamino Acid Derivatives ^a

Bpoc-L-amino acids	mp (°C)	$[\alpha]_D^{25}$ (c = 1, methanol)
Ala	108–111	−27.2°
Ala · DCHA	178–180	−39.4° ^b
Arg(NO ₂) · etherate ^c	120–123	+6.3°
Asn	218–220	−13.8° ^d
Asp(OBzl)	186–189	+3.4°
Cys(Meb) · DCHA	168–169	−9.1°
Glu	195–198	−8.8°
Glu(OBzl) · CHA	147–149	+20.9°
Gln	140–142	+3.9°
Gln · CHA	172–173	+11.2° ^b
Gly	131–132	—
His	146–149	−13.0° ^d
His(Bzl)	186–188	−4.3° ^d
His(Boc)	133–134	+21.0°
Ile	215–218	−14.5°
Leu	227–230	−12.0°
Lys(Z)	Amorphous	−1.8°
Met(O) · DCHA	160–161	+15.0°
Phe · CHA	116–119	+33.0°
Phe · DCHA	106–108	+29.2° ^b
Phg · CHA	176–178	−48.1°
Ser · DCHA	154–156	+2.5° ^b
Ser(Bzl) · CHA	155–156	+2.1°
Ser(Bu') · CHA	180–181	+21.0°
Ser(Bu') · DCHA	181–183	+21.3° ^b
Thr · CHA	154–156	−0.6° ^b
Thr(Bzl)	148–150	+11.3°
Trp	142–145	+36.8°
Tyr(Bzl)	198–200	+12.2°
Val	223–225	−14.9°
Val · CHA	178–180	−8.0°
Val · DCHA	179–180	−6.9° ^b

^a Data from Sieber and Iselin (1968a,b), Wang and Merrifield (1969b), Schnabel *et al.* (1971b), Yamashiro *et al.* (1972).^b $[\alpha]_{578}$.^c Analyzed for 1 equivalent of diethyl ether.^d c = 1, Dimethylformamide.

c. *Other N α -Protected Amino Acid Derivatives.* The preparation of *o*-nitrophenylsulfenylamino acids from *o*-nitrophenylsulfenyl chloride has been described by Zervas *et al.* (1963) and Zervas and Hamalidis (1965). Physical constants and tables of many side-chain protected Nps-amino acids can be found there (see Merrifield, 1969a).

2-Benzoyl-1-methylvinyl (Bmv) amino acids (99) were first synthesized by Dane *et al.* (1962) as potassium salts which were unstable, hygroscopic, and insoluble in methylene chloride or chloroform. Southard *et al.* (1971a,c) described the ready synthesis of Bmv-amino acids as dicyclohexylammonium salts which are nonhygroscopic, stable, soluble in several solvents, and which can be prepared in high yield and purity.

d. *Improved Preparations of Various Side-Chain Protected Amino Acid Derivatives.* Nitroarginine: Lenard (1967) described quantitative formation of nitroarginine by treatment of the amino acid with KNO₃ in hydrogen fluoride for 30 minutes at 0°. The crystalline product was isolated in 85% yield.

O-Benzyl-Boc-serine: This has been directly prepared from Boc-serine by treatment with sodium in liquid ammonia followed by benzyl bromide (Hruby and Ehler, 1970).

O-Benzylthreonine: Treatment of threonine with benzyl alcohol and *p*-toluenesulfonic acid in toluene at reflux with azeotropic removal of water for 20 hours, followed by alkaline saponification of the product affords *O*-benzylthreonine in 16% overall yield (Mizoguchi *et al.*, 1968).

D. COUPLING METHODS

Requirements for peptide bond formation in solid-phase synthesis are: (a) quantitative and rapid reaction, (b) no undesirable side reactions, and (c) minimal racemization.

In his review Merrifield (1969a) noted that most of the conventional peptide coupling reagents have been used at least once in solid-phase peptide synthesis, but only two have been used extensively. The two procedures referred to are the dicyclohexylcarbodiimide (Sheehan and Hess, 1955) and the *p*-nitrophenyl ester (Bodanszky, 1955, 1957) couplings, and they have continued to be the methods of choice during the past few years.*

1. Dicyclohexylcarbodiimide

The carbodiimide method of Sheehan and Hess (1955) has become by far the most frequently used procedure for solid-phase synthesis.* The con-

* The use of DCCI amounts to about 92% of all solid-phase syntheses (until November 1969, as reported by Marshall and Merrifield, 1970) and the use of active esters to about 6%, leaving about 2% for all other methods.

venience of its use, not requiring separate activation of the carboxyl component but only a simple addition to a mixture of acid and amino components, rendered it ideally suitable for the solid-phase procedure. The main reason, however, for the success of the carbodiimide method has been its high reactivity, even in the presence of water. In addition, the ready commercial availability of dicyclohexylcarbodiimide has certainly been an important factor. Comprehensive tables listing the applications of solid-phase peptide synthesis have been compiled by Merrifield (1968a, 1969a, Table V), by Losse and Neubert (1970a), and by Marshall and Merrifield (1970). Most of the applications of dicyclohexylcarbodiimide follow the "standard procedure" described by Marshall and Merrifield (1965), some with minor alterations. In the following account those works will be discussed in which significant modifications of the standard procedure have been made and evaluated.

a. Modified Procedures. The solubility of some amino acid derivatives in methylene chloride is limited and dimethylformamide has frequently been used although carbodiimide couplings do not proceed as well in dimethylformamide (Wieland *et al.*, 1969a). Suspensions of amino acid derivatives can apparently be used as shown by Jorgensen *et al.* (1970a) who successfully employed a suspension of *N* α -*tert*-butyloxycarbonyl-*im*-benzylhistine in a 1:1 mixture of dimethylformamide-methylene chloride in a synthesis of an angiotensin analog.

Southard *et al.* (1969) effectively used solutions of dicyclohexylammonium salts of *N* α -(2-benzoyl-1-methylvinyl)amino acids in methylene chloride in the presence of equimolar amounts of anhydrous *p*-tolenesulfonic acid in dicyclohexylcarbodiimide mediated solid-phase synthesis. The possibility of using dicyclohexylammonium salts directly, without prior conversion into free acids, is an advantage since amino acid derivatives with acid labile *N* α -protecting groups (such as *tert*-butyloxycarbonyl-, *o*-nitrophenylsulfenyl-, 2-(*p*-biphenyl)isopropylloxycarbonyl-, or 2-benzoyl-1-methylvinylamino acids) are frequently prepared and stored as dicyclohexylammonium salts (Klieger *et al.*, 1961).

So-called adsorption coupling was examined by Esko and Karlsson (1970). Boc-amino acids or Boc-peptides (2–6 moles/mole resin-amino groups) in methylene chloride were incubated for 4 hours at room temperature to form salts with the resin-bound amino groups.* Excess Boc-amino acid or Boc-peptide was then removed by washing with methylene chloride, followed by addition of carbodiimide in methylene chloride. Although adsorption coupling did not show any advantage over the standard Merrifield

* Salt formation between amine and carboxyl components prior to dicyclohexylcarbodiimide condensation in solution synthesis has been examined by Taschner *et al.* (1965).

procedure in a synthesis of a hexapeptide using Boc-amino acids, racemization appeared to be suppressed when Boc-peptides were coupled. When the adsorption coupling was carried out at low temperatures (-12° to $+4^{\circ}$), higher yields than at room temperature were obtained in couplings of Boc-Ile or Boc-Leu-Ala to Val-resin. Elliott *et al.* (1973), who independently studied adsorption coupling during syntheses of several bradykinin analogs, observed that purer products requiring "less involved purification procedures" were obtained in this way, as compared to the standard solid-phase procedure in which excess acylamino acid is present during the coupling stage.

b. The Problem of Incomplete Reaction. The problem of achieving quantitative reaction has received the widest attention, especially since complete coupling in each step is one of the basic imperatives of the method by its very design. The consequences of failing to achieve full acylation, that is formation of deletion sequences leading to inseparable microheterogeneous products, have been discussed frequently (Merrifield, 1963, 1968b; Bayer, 1970; Bayer *et al.*, 1970b); see Section II,C.

The overall record of solid-phase synthesis to date attests to the obvious fact that complete reaction throughout all cycles cannot, at present, generally be achieved. An illustrative example has been recorded by Bayer *et al.* (1968a) who followed the progress of a synthesis of Apoferrodoxin from *Clostridium pasteurianum* by titrating the amounts of free amino groups after deprotection in each cycle (Fig. 10). The level of amino groups decreased steadily and finally dropped to 38% of the starting value after fifty-four cycles. A more rapid decline was observed by Jollès and Jollès (1968) during a synthesis of the octapeptide derivative, Boc-Cys(Bzl)-Asn-Asp(OBzl)-Gly-Arg(NO₂)-Thr-Pro-Gly-Resin (sequence 64–71 of hen egg-white lysozyme). Amounts of amino acid incorporation, as determined by amino acid analysis after each coupling (taking Gly as 1.0), were 0.8 Pro, 0.6 to 0.5 Thr through Asn, and 0.3 Cys(Bzl).

Obvious causes for the observed defects are (a) loss of peptide from the solid support, (b) incomplete deprotection, (c) chain termination, and (d) incomplete coupling, each of which can occur singly or combined. The latter three faults result in formation of either truncated or failure sequences, as discussed in Section II,C.

Experimental evidence for the formation of truncated sequences was obtained by Beyerman (1972) who separated, by Sephadex LH-20 chromatography, a nonapeptide derivative (sequence 24–32) from a solid-phase preparation of human calcitonin sequence 11–32. Young *et al.* (1972) isolated an equimolar amount of truncated undecapeptide from a pentadecapeptide preparation of tobacco mosaic virus-protein-(98–112).

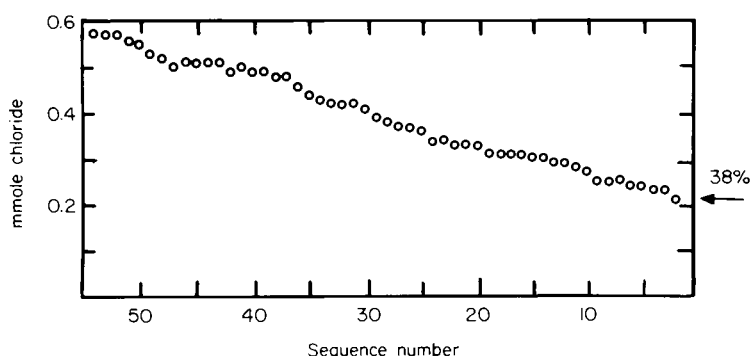


FIG. 10. Titration of triethylamine hydrochloride during a solid-phase synthesis of apoferritin showing the decreasing free amine content after each deprotection step. From Bayer *et al.* (1968a).

Experimental evidence for the formation of failure sequences in solid-phase synthesis has recently been accumulated by Bayer and collaborators (1970a; Bayer, 1970) who set out to search for these "guerilla" peptides in a specifically designed trap. To increase the amount of detectable failure sequences, a repeating sequence of the type $(A-B)_n$ was chosen as the synthetic goal in which the small amounts of individual amino acid omissions would accumulate during the repetitive chain elongation in the form of sequence A-A or B-B. The dodecapeptides $(Leu-Ala)_6$ and $(Ala-Phe)_6$ were synthesized. Partial hydrolysis of the final products followed by trifluoroacetylation of peptides, gas-chromatographic separation, and mass spectrometric identification showed the presence of failure sequences (Table XIII). Leu-Leu (2.6%) and Ala-Ala (1.4%) were found. As much as 9.3%

Table XIII—Occurrence of Failure Sequences in Solid-Phase Syntheses of Dodecapeptides (Determined by Gas Chromatography and Mass Spectrometry after Partial Acid Hydrolysis)^a

Partial Hydrolysate of $(Leu-Ala)_6$			Partial Hydrolysate of $(Ala-Phe)_6$	
Peptide	Amount (%)	Amount acetylation after coupling, (%)	Peptide	Amount (%)
Ala-Ala	1.4	0.1	Ala-Ala	Not detectable
Leu-Ala	65.5	70.0	Phe-Ala	39.7
Ala-Leu	28.5	29.3	Ala-Phe	51.0
Leu-Leu	2.6	0.1	Phe-Phe	9.3

^a After Bayer, 1970 and Bayer *et al.*, 1970a.

Phe-Phe was detected after partial hydrolysis of the (Ala-Phe)₆ peptide, but no Ala-Ala was present possibly due to nonrandom hydrolysis. Average coupling yields of slightly over 99% per step were indicated by these results. A deletion peptide lacking tyrosine has been isolated by Jorgensen *et al.* (1971c) during purification of [Asn¹,Pro⁵]-angiotensin II.

Failure sequences in oligamino acids give rise to shortened peptides. In a solid-phase synthesis of hepta- γ -glutamic acid (Meienhofer *et al.*, 1970a) using Boc-glutamic acid α -benzyl ester, the product was analyzed by high voltage electrophoresis showing the presence of all six lower homologs (Fig. 11). Solid-phase synthesis of heptalysine via *N* α -*tert*-butyloxycarbonyl-*N*-trifluoroacetyllysine (Anfinsen, 1968) gave a product that was shown by ion exchange chromatography to contain 82% of the heptamer and 18% of materials containing hexalysine and lower oligomers. This result is compatible with a coupling efficiency of approximately 98% at each step.

There can be no doubt that amino acid omission poses a serious problem in solid-phase synthesis. It should be remembered that yields of 98% per step are of no value for syntheses of large peptides containing over fifty amino acid residues (see Section II,C).

Several factors have been implicated as influencing rates and yields of reactions in solid-phase peptide synthesis. They are: (1) the nature (bulkiness) of amino acid side chains, (2) the peptide chain length, (3) the sequence of the synthetic peptide, (4) the polarity of solvents, and (5) the degree of cross-linking of the resin.

Observations that peptide bonds involving amino acids with *bulky side chains* are not formed as readily as others have been reported, e.g., by Weygand and Obermeier (1968), Esko *et al.* (1968), and Dorman (1969). Particularly difficult to incorporate are Boc-isoleucine, Boc-valine,

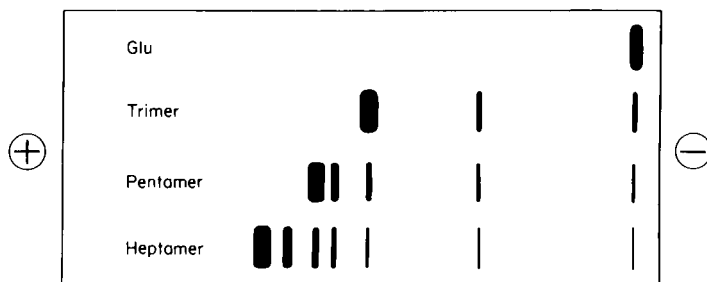
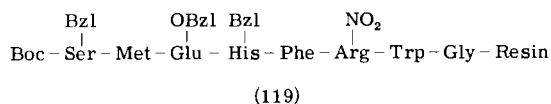
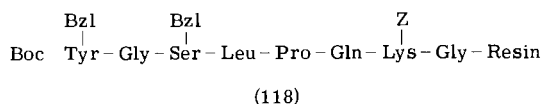


FIG 11. High voltage paper electrophoresis of γ -linked glutamic acid oligomers prepared by solid-phase synthesis using Boc-Glu-OBzl (Meienhofer *et al.*, 1970a). The Boc groups were cleaved with 4 N HCl in dioxane. Electrophoresis was carried out on Whatman 3 MM paper (3400 V, pH 2.1, 1 hour, ninhydrin staining).

Boc-leucine, Boc-tyrosine benzyl ether, and, sometimes, Boc-glutamine. The influences of *chain length* and of steric hindrance on the extent of the coupling reactions in a solid-phase synthesis of the dodecapeptide (Leu-Ala)₆ have been studied by Hagenmaier (1970). The first condensation proceeded with 99.5% yield, and with 100% after a repeat with fresh Boc-amino acid and dicyclohexylcarbodiimide. The next four couplings gave 96–99.5% yields after one repetition of the condensation reaction. Thereafter, the yields decreased steadily to as low as 65–75%, and second repetitions did not increase the yield. Acetylation was effective for unreacted alanine amino groups but much less for leucine, indicating that steric effects were reducing the accessibility of free amino groups. Geising and Hörnle (1972) also observed marked gradual decreases of coupling speed with increasing chain length when the periodic formation of the same peptide bond was compared during the synthesis of the sequence oligomer (Ile-Val-Leu-Ala)₅.

Experimental evidence for a *sequence dependence* on coupling rates has been presented by Geising and Hörnle (1973) who coupled Boc-isoleucine to two undecapeptides with N-terminal valine but with sequence differences in their center parts. Sudden dramatic drops in coupling yield of an amino acid residue after a series of successful cycles have also been observed. The first recorded case (Merrifield, 1967) occurred during a synthesis of angiotensinylbradykinin, the progress of which was followed by amino acid analysis at each cycle (Fig. 12). Changing the solvent in the deprotection step, from acetic acid to dioxane, and using a less cross-linked resin (1%) eliminated the shortcoming. Chou *et al.* (1971) experienced incomplete couplings in the last two cycles (30–50% yields and 5–10% yields, respectively) of two different octapeptide sequences (118 and 119).



These failures were due to incomplete deprotection prior to the condensations. Complete reaction in these and related peptides was again achieved by solvent changes and addition of urea to increase solvent polarity. Westall

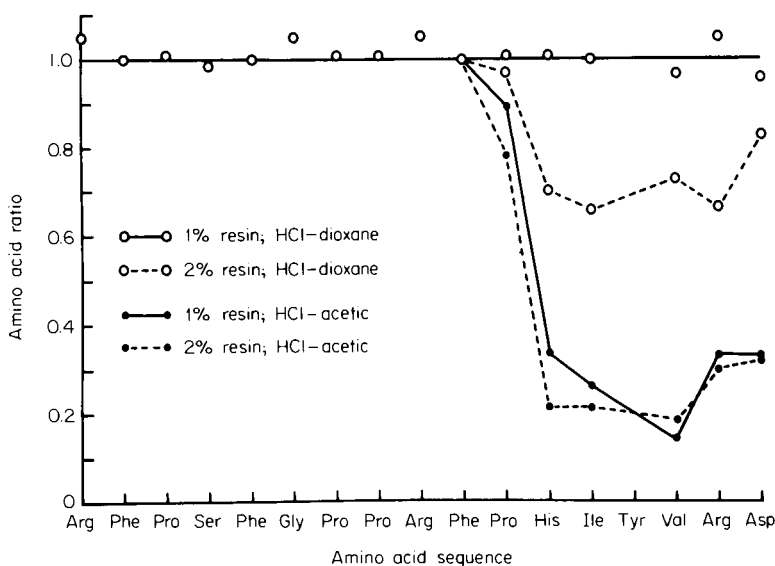


FIG. 12. Sudden decrease in amino acid incorporation during a solid-phase synthesis of angiotensinylbradykinin. Effects of solvent and resin cross-linking on the occurrence of this failure are shown. The synthesis proceeded in a stepwise manner from the C-terminal Arg shown on the left toward the N-terminal Asp on the right. Coupling yields were determined by amino acid analysis after each cycle. From Merrifield (1967).

and Robinson (1970) discovered a pronounced solvent effect* on the coupling yield with a particularly difficult amino acid condensation (glutamine) (Fig. 13). A mixture of dimethylformamide and methylene chloride (3:7) and the presence of 1.5 *M* urea remedied the failure.

A more general solvent dependence in carbodiimide couplings has already been examined by Merrifield (1963). Methylene chloride and dimethylformamide were found to be satisfactory while benzene, dioxane, ether, ethyl acetate, and ethanol were not useful (Merrifield, 1969a). Comparison between methylene chloride and dimethylformamide as coupling solvents in two syntheses of the linear decapeptide precursor of antamanide, H-Phe-Pro-Pro-Phe-Phe-Val-Pro-Pro-Ala-Phe-OH, under otherwise identical conditions showed that methylene chloride gave a significantly better result (Wieland *et al.*, 1969a). It was also pointed out in this report as well as in many others (e.g., Polzhofer and Ney, 1970a; Wieland *et al.*, 1971c) that the purity and dryness of solvents are decisive factors for obtaining satisfactory results with solid-phase syntheses (see Section IV,B). Even analytic or

* Marked solvent dependence was also observed in hydrolysis of resin-bound amino acids (lysine) (Scotchler *et al.*, 1970).

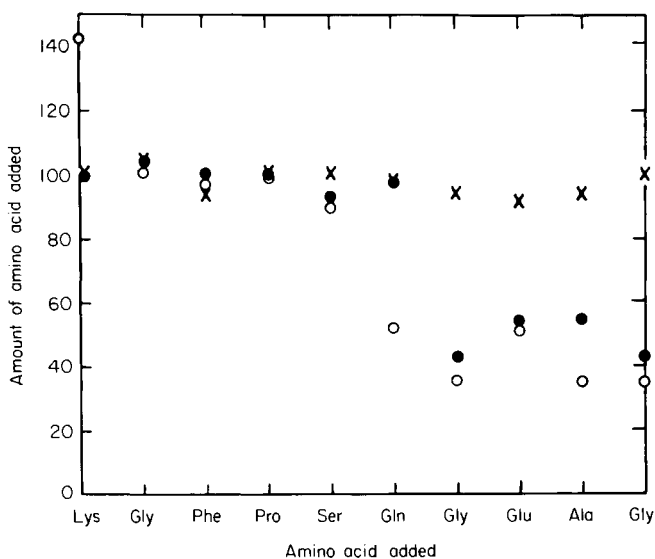


FIG. 13. Solvent dependence of chain termination (incomplete Boc-glutamine incorporation) during solid-phase syntheses of Gly-Ala-Glu-Gly-Gln-Ser-Pro-Phe-Gly-Lys-Resin. Reagents and solvents used were: ○, 1 N HCl in acetic acid (containing 1% mercaptoethanol) for deprotection, DCCI in methylene chloride for coupling (6 hours); ◐, trifluoroacetic acid in methylene chloride (1:1, containing 5% mercaptoethanol) for deprotection, DCCI in methylene chloride for coupling (6 hours); X, trifluoroacetic acid in methylene chloride (1:1, containing 5% mercaptoethanol) for deprotection, DCCI in dimethylformamide-methylene chloride (3:7) (6 hours). From Westall and Robinson (1970).

reagent grade solvents are often not sufficiently pure for use without prior distillation.

Sheppard (1973) has offered an explanation why amino acid bulkiness, peptide chain length and sequence, solvent polarity, and resin density would influence amino acid incorporation in the observed ways. The polymer support with its growing peptides might be regarded as a graft copolymer consisting of backbone and pendant chains of widely different polarities. Figure 14B shows such a peptide-resin system with both the nonpolar polystyrene backbone and the shorter, more polar peptide chains partially extended. In a nonpolar medium the peptide chains may collapse while the backbone becomes very extended (Fig. 14A). In a polar solvating medium the hydrocarbon backbone may collapse entangling in it the (extended) peptide chains (Fig. 14C). Both in A and C the terminal free amino group of some peptide chains might become buried or hindered. The balance between situations A, B, and C and other intermediate phases may be delicate and very

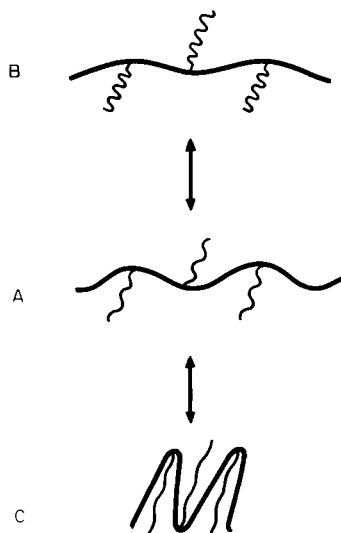


FIG. 14. Swelling and shrinking of peptide-resin. The polymer backbone carrying the growing peptide chains is depicted as a heavy line. A, Peptide chains and polystyrene strands partially extended; B, collapse of peptide chains in nonpolar solvating medium; C, collapse of supporting resin strands in polar solvating medium. From Sheppard (1973).

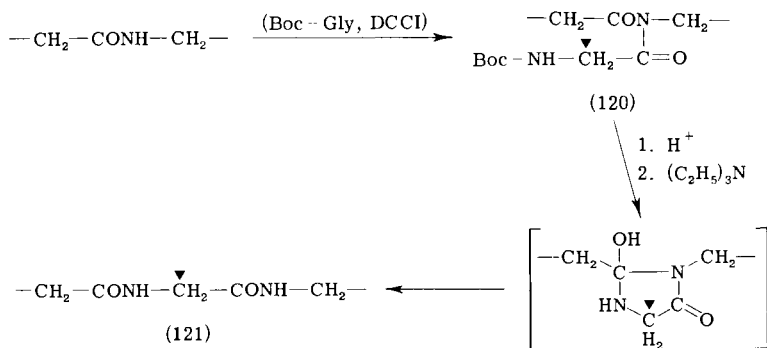
dependent upon the sequence and conformation of the peptide.

Efforts have been made to improve the coupling efficiency within the presently existing framework of solid-phase strategy. Obvious experimental approaches to improve incomplete carbodiimide coupling include: (a) longer reaction time, (b) larger excesses of the soluble component and reagent, (c) repeated reaction, and (d) variations of solvent, temperature, mode of stirring, etc.

The application of increasingly *longer reaction times* has been a noticeable trend. The standard 2-hour period which Marshall and Merrifield (1965) had originally recommended has gradually become extended to 4, 5 (e.g., Gutte and Merrifield, 1969, 1971), or more hours. Bayer *et al.* (1971b) believe that one can no longer advocate any "standard" reaction time which could be safely applied in routine fashion throughout a new synthesis. Since it is a priori unknown and not always predictable which particular peptide bond(s) might be formed at slow rate, many investigators have adapted as a safety factor rather longer reaction periods than actually are required for most couplings. Thus, reactions are often allowed to proceed overnight (15 hours). In some instances even longer reaction periods (24 hours) have been applied (e.g., Sano and Kurihara, 1969). Experimental

evidence indicated that periods as long as 24 hours might, indeed, sometimes be required (Esko *et al.*, 1968; Weygand and Obermeier, 1968). However, most couplings are probably complete in less than 30 minutes, and this can now be easily determined by the very rapid and sensitive ninhydrin color test (Kaiser *et al.*, 1970) on very small samples (5–15 mg).

Application of *larger excesses* of Boc-amino acids and carbodiimide than the generally used three- to four fold amounts relative to the resin substitution would seem to be obvious remedies for incomplete coupling. Amounts of 30- to 70-fold have been used in routine manner through the final eighteen steps of a solid-phase synthesis with horse heart cytochrome c as target sequence (Sano and Kurihara, 1969). However, improved coupling yields were not obtained when rather large excesses were applied in valinomycin syntheses (Losse and Klengel, 1971). It has, on the contrary, to be borne in mind that very large excesses of acylating component and reagent could cause side reactions, such as diacylimide formation (Wieland and Heinke, 1956; Kopple and Renick, 1958) and aminoacyl insertion (Wieland and Mohr, 1956; Brenner and Wehrmüller, 1957). That such side reactions can, indeed, occur has been shown by Mitchell and Roeske (1970) who detected a small amount of tetraglycine (0.2%) in a preparation of triglycine when excesses of 22 equivalents of Boc-glycine and dicyclohexylcarbodiimide were used. Thus, diacylimide formation (120) followed by amino acyl insertion (121) can occur when very large excesses are used especially with Gly-Gly sequences.



Repeated carbodiimide coupling to achieve more complete reaction was first suggested by Weygand and Obermeier (1968) and has been found necessary in several steps during syntheses of angiotensin II analogs (Jorgensen *et al.*, 1969) and of encephalytogenic peptides (Shapira *et al.*, 1971) when more than 5% of resin-amino groups remained unacylated. This procedure has recently been used in routine fashion by several investigators (e.g.,

Manning *et al.*, 1970; Hancock *et al.*, 1971). Other authors (Yajima *et al.*, 1970a) use repeated coupling for amino acids with bulky side-chains (*e.g.* valine or leucine) that are known to give, sometimes, incomplete reaction.

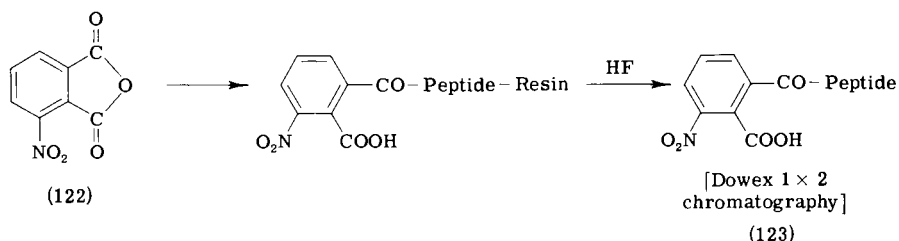
Another stratagem suggested for reducing the number of peptide chains that could become inaccessible or buried during the progress of a synthesis is to start with a very low level of C-terminal amino acid substitution. An additional suggestion is use of the most hindered C-terminal amino acid in syntheses of cyclic peptides, when the C-terminal is an object of choice. Both proposals were tested in syntheses of valinomycin (Losse and Klengel, 1971).

Since 100% complete coupling cannot generally be taken for granted and because it is very important that no unreacted amine remains (which would be liable to react with the subsequent *tert*-butyloxycarbonylamino acid resulting in failure sequence formation) acylation with potentially more reactive agents has been employed to block off irreversibly any remaining amino groups. Acetylation with a large excess of acetic anhydride in dimethylformamide in the presence of triethylamine for several hours at room temperature was investigated by Merrifield (1963). The amine content of a valine resin was reduced from 0.72 to 0.0029 mmole/gm, thus leaving about 0.4% amine unreacted. The amount of 0.4% amine remaining after acetylation might be the lowest attainable limit and is probably lower than residual amine remaining after the incorporation of *tert*-butyloxycarbonylamino acids. In a model system acetylation was found to reduce failure sequence formation considerably, see Table XIII (Bayer *et al.*, 1970a). Acetylation after each coupling was advantageous in syntheses of a heptapeptide (Blake and Li, 1968). Consequently, acetylation with the aid of acetic anhydride has been used in several solid-phase syntheses, particularly of large peptides (see, *e.g.*, Hancock *et al.*, 1971; Marshall *et al.*, 1973). Weber *et al.* (1969) acetylated with four-fold molar excesses (per mole peptide) of glacial acetic acid and dicyclohexylcarbodiimide in methylene chloride.

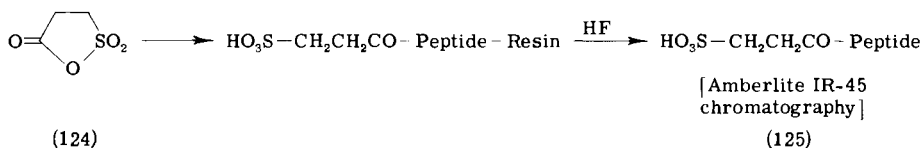
In a comparative study of "*terminating agents*" Markley and Dorman (1970) set forth a number of criteria for suitable reagents. Ideally, a terminating reagent must (*a*) have a low steric hindrance for reaction at less accessible resin sites, (*b*) have high reactivity towards amino groups, yet have no effect on peptide bonds or other functional groups, (*c*) form a covalent bond with the amino group to be terminated that is stable to all reaction conditions used subsequently in the synthetic procedure, and (*d*) facilitate separation of terminated amino acid or peptide fragments from the final target peptide after removal of the solid support. Two reagents, methanesulfonyl chloride in the presence of triethylamine (Truce *et al.*, 1964) and acetyl-imidazole (Staab, 1962) were found to merit a more detailed study. Using

Leu-Ala-Gly-Val as a model system and acetic anhydride-triethylamine as a basis for comparison, *N*-acetylimidazole exerted the most efficient termination. It would be interesting to examine the effectiveness of this reagent in systems (Hagenmaier, 1970) where acetic anhydride-triethylamine failed.

To facilitate the separation of truncated *N*-acylpeptides from the final product after removal from the solid support, Wieland and collaborators (1969b) proposed the use of *o*-nitrophthalic anhydride (122) for the acylation of remaining free amine. Truncated *N*-(2-carboxy-3-nitrobenzoyl) peptides (123) are produced. Separation of these peptides by ion exchange

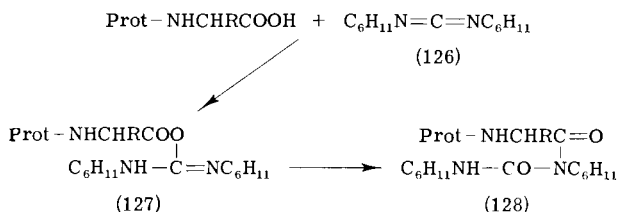


chromatography should be facilitated by the additional negatively charged carboxyl group (acidic handle); see Section III,F,2. Wissmann and Geiger (1970) described the use of 3-sulfopropionic anhydride (124) to give *N*-(3-sulfopropionyl)peptides (125). This reagent has the advantage of being

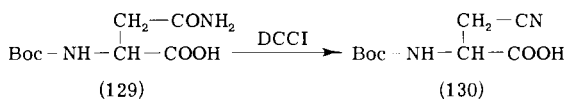


considerably smaller and should penetrate faster into the resin and reach less accessible sites. However, Schaich and Schneider (1973) observed unfavorable side effects when 3-sulfopropionic anhydride (124) was used in a synthesis of a heptacosapeptide. The overall effect of successful termination of residual amine is, of course, conversion of potential sources of failure sequences into truncated sequences which are fewer in numbers and more easily separable (see Section II,C).

c. Side Reactions. Well-known side reactions involving the use of dicyclohexylcarbodiimide (126) in solution synthesis are (a) formation of *N*-acylurea (128) (Sheehan *et al.*, 1956; Merrifield and Woolley, 1956; Zahn and Diehl, 1957a,b; Buzas *et al.*, 1961) by an intramolecular rearrangement of the acylating *O*-acyl intermediate (127), and (b) nitrile formation (130) from carboxamide groups during activation of glutamine or aspara-



gine (129) derivatives (Ressler, 1956; Gish *et al.*, 1956; Paul and Kende, 1964). *N*-Acyl-*N,N'*-dicyclohexylurea formation was found to occur in sol-



id-phase synthesis (Merrifield, 1969a). Its detrimental effect is to consume part of the Boc-amino acid and the reagent, but the side product does not become part of the peptide and subsequently is removed during washings. The side reaction is fast in dimethylformamide but slow in methylene chloride. Rapid consumption of Boc-proline and DCCI in a solid-phase synthesis of bradykinin was observed when dimethylformamide was used as a solvent. Up to 8 moles of Boc-proline and reagent had to be used in this case (Merrifield, 1964b). With methylene chloride as a solvent the acylurea formation was so decreased that the use of 1.5 moles of Boc-proline gave a quantitative coupling. *N*-Hydroxysuccinimide (Wünsch and Drees, 1966; Weygand *et al.*, 1966) and 1-hydroxybenzotriazole (König and Geiger, 1970a) are known to suppress *N*-acylurea formation in solution syntheses. Thus far, the applications of the DCCI-HOSu procedure to solid-phase synthesis (Weygand and Ragnarsson, 1966; Omenn and Anfinson, 1968; Noda *et al.*, 1970b; Windridge and Jorgensen, 1971a,b) have shown somewhat equivocal results.

Nitrile formation in Boc-asparagine and Boc-glutamine is potentially much more dangerous since the resulting β -cyanoalanine or γ -cyanoaminobutyric acid residues would become part of the growing resin-linked peptides. However, this danger has always been by-passed by the general use of active esters for the incorporation of asparagine and glutamine; and the extent to which nitrile formation does actually occur in solid-phase synthesis has not been investigated thus far. It is known from solution synthesis that dehydration of the carboxamide group to a nitrile group occurs only during the coupling. Once the glutamine and asparagine residues are part of peptide chains, they seem to be resistant to the action of carbodiimide (Bod-

anszky and Ondetti, 1966). The danger of diacylimide formation has been discussed above (120) and should normally be minimal.

d. Racemization. Freedom from racemization is an imperative prerequisite for solid-phase synthesis which is equally important to that of 100% complete coupling. Arguments similar to those discussed in connection with failure sequence formation due to incomplete reaction also apply to diastereoisomer formation as a consequence of racemization. Thus, assuming a 0.2% rate of racemization per step, a synthetic preparation containing 124 amino acid residues, such as ribonuclease A, would be about 88% optically pure, and 12% of the product would consist of a microheterogeneous mixture of diastereoisomers. For a peptide consisting of 190 amino acid residues (e.g., human growth hormone), the diastereoisomer mixture would amount to approximately 17%. Separation of diastereoisomer contaminants from the desired optically pure product would be extremely difficult and certainly incomplete even with the most powerful fractionation procedures presently available, a problem very similar to that of failure sequence removal.

The standard experimental Merrifield procedure represents a system with minimal danger of racemization to the best available knowledge and experience. Peptide chain elongation by single amino acid residues possessing *N* α -urethane-type protecting groups and using dicyclohexylcarbodiimide can be expected to show ordinarily no measurable racemization. Nevertheless, many efforts have been made to provide experimental evidence for the assumed absence of racemization in solid-phase synthesis.

The overall experimental evidence obtained from a variety of different assay techniques including optical rotation, enzymic digestion (Merrifield, 1963), chromatographic procedures, gas chromatography (see, e.g., Bayer *et al.*, 1970b), and biological testing led Merrifield (1969a) to conclude that "within the limits of these methods . . . no racemization has been detected in any peptide made by the standard, stepwise solid-phase method. . . ." However, some of these tests were done on purified products, and absence of diastereomeric peptides in purified products is, of course, not a criterion that racemization did not occur during synthesis. For example, 15% D-benzylhistidine was formed during syntheses of angiotensin II analogs, but chromatographed products contained no D-histidine (Jorgensen *et al.*, 1971b). Moreover, the sensitivity limits of most racemization tests have been well over 0.5% racemate detection. Only recently have more sensitive methods been developed to detect as little diastereoisomer formation as 0.2% (Manning and Moore, 1968) or even ± 0.001 to $\pm 0.05\%$ (Kemp *et al.*, 1970a,b). At these sensitivity levels even those synthetic methods,

such as the azide method (Curtius, 1902) which previously had been regarded as "racemization-free," were found to be deficient. Indeed, under adverse circumstances as much as 10% diastereoisomer formation (20% racemization) was observed in a peptide fragment condensation using the azide method (Sieber *et al.*, 1970). It has now been generally agreed that a "racemization-free" coupling method does not exist, and that even with the most scrupulous efforts it can only be attempted to keep racemization at a low level.

Returning to consideration of undesired occurrence of racemization in solid-phase synthesis, there have been a few examples demonstrated experimentally. Inukai and collaborators (1968) found during syntheses of peptides related to physalaemin (Erspamer *et al.*, 1964) about 3% racemate formation by the Bodanszky test (Bodanszky and Conklin, 1967). Some racemization was observed at one position in an insulin A-chain synthesis (Merrifield and Marglin, 1967) indicating a certain sequence influence on the degree of racemization. Considerable racemization (20%) of *N* α -*tert*-butyl-oxycarbonyl-*N*^{im}-benzylhistidine during syntheses of angiotensin II analogs was detected (Jorgensen *et al.*, 1970a) and its degree was determined by assays using L-amino acid oxidase.

Racemization studies (Windridge and Jorgensen, 1971a) with a model system, Boc-His(Bzl)-Glu(OBzl)-resin, showed formation of 11, 14, and 25% of D-histidine following carbodiimide couplings in methylene chloride, pyridine, and dimethylformamide, respectively. A coupling of *N* α -*tert*-butyloxycarbonyl-*N*^{im}-benzylhistidine to the Glu(OBzl)-resin using *N*-hydroxysuccinimide-dicyclohexylcarbodiimide in methylene chloride gave, in this instance, a favorable suppression of racemization to below 0.2%. The yield was low (70%), and β -alanine, a known side product of the reaction between dicyclohexylcarbodiimide and *N*-hydroxysuccinimide (Gross and Bilk, 1968a, b; Weygand *et al.*, 1968b) was found in hydrolysates. More recently it was shown that 1-hydroxybenzotriazole (König and Geiger, 1970a) is effective in reducing racemization during solid-phase peptide synthesis with *N* α -*tert*-butyloxycarbonyl-*N*^{im}-benzylhistidine to low levels (0.5%) without impairing coupling efficiency (Windridge and Jorgensen, 1971b). In this context a long-standing observation should be remembered. Anderson and Callahan (1958) observed drastic lowering of racemization in carbodiimide (fragment) condensations by working at 0°. Pettit and Jones (1971) have described a solid-phase synthesis in which they did apply ice cooling during the addition of dicyclohexylcarbodiimide.

The potential presence of microheterogeneous mixtures of diastereoisomers in peptides prepared by the solid-phase method should always be borne in mind when biological activities of such preparations are interpreted.

ed. While many components containing one D-amino acid residue might simply be inactive or of similar activity as the target product, some might possess higher or protracted activity (e.g., [D-Ser¹, Nle⁴, Val-NH₂²⁵]- β -corticotropin-(1-25) (Boissonnas *et al.*, 1966), or [D-Ser¹, Lys¹⁷, Lys-NH₂¹⁸]- β -corticotropin-(1-18) (Desaulles *et al.*, 1969)] while others might possess inhibitory activity (e.g., [D-His⁶]- α -melanotropin-(6-10) (Yajima and Kubo, 1965a,b)).

In conclusion:

- a. It should not be assumed that solid-phase peptide synthesis proceeds generally without any racemization.
- b. Detectable racemization (>0.2%) occurs infrequently.
- c. Occurrence of detectable racemization is a priori unpredictable and depends on circumstances that differ from one individual synthesis to the next. Routine checking for racemization is, therefore, advisable.
- d. Low levels of racemization (<0.2%) can potentially occur in every peptide coupling.
- e. Solid-phase preparations containing large numbers of amino acid residues (>50) might be contaminated by considerable amounts of microheterogeneous diastereoisomer mixtures as a consequence of low levels of racemization.

2. Active Esters

Active esters have been used successfully in solid-phase synthesis. Pertinent examples, in which *p*-nitrophenyl esters (Bodanszky, 1955) have been used, are syntheses of the C-terminal hexapeptide of secretin (Bodanszky and Sheehan, 1966), of bovine insulin A-chain (Hörnle, 1967), and of [Ala⁶, Ala¹¹]-ovine insulin A-chain (Weber *et al.*, 1968). The principal advantages of active esters over carbodiimide for solid-phase synthesis are (a) they are mostly stable, well-defined (often crystalline), easily available preactivated substances that are simply added to the reaction vessel not requiring any additional reagent, (b) they allow spectrophotometric monitoring of the progress of coupling (Hörnle, 1967; Rudinger and Gut, 1967), and (c) unreacted excesses of active esters can be recovered. Their most serious disadvantage is considerably slower reaction (Karlsson *et al.*, 1970a) forcing, in general, the provision of 5–20 times longer reaction periods. Even then, couplings often remain rather less complete than those mediated by dicyclohexylcarbodiimide in identical systems (Weygand and Obermeier, 1968; Klostermeyer *et al.*, 1967; Klostermeyer, 1968; Visser *et al.*, 1968). In a synthesis of bovine insulin A-chain (Hörnle, 1967), *p*-nitrophenyl esters have been used exclusively, and the average yield per step was 94% through twenty cycles. Karlsson *et al.* (1970a) thoroughly examined

the reactivity of *p*-nitrophenyl esters in solid-phase synthesis, and they concluded that these react slowly even when present in very large excess, and, that by comparison, carbodiimide gives faster and more complete coupling (see, also, Maher *et al.*, 1971). Nevertheless, *p*-nitrophenyl esters are generally used for the incorporation of asparagine and glutamine residues, because dicyclohexylcarbodiimide causes nitrile by-product formation (130) from the carboxamides, as discussed above.

The additional operational convenience offered by the use of active esters for solid-phase synthesis led to many investigations into the usefulness of other active esters. Earlier studies have been reviewed by Merrifield (1969a). More recently Klostermeyer (1968) reported a complete lack of success when *N*-hydroxysuccinimide esters (Anderson *et al.*, 1963) were applied in an attempted synthesis of gramicidin S; while Anfinsen (1968) used these esters successfully in a preparation of a hexapeptide. Pentachlorophenyl esters have also been used in several syntheses (Johnson and May, 1969; Visser and Kerling, 1970; Visser *et al.*, 1968, 1970a,b; Yajima *et al.*, 1970b). Bodanszky and Bath (1969) compared rates of reactions of different active esters of Boc-leucine with resin-bound glycine and valine and with glycine *tert*-butyl ester and valine *tert*-butyl ester in solution (Table XIV). It was found that the *o*-nitrophenyl ester reacts 4–5 times faster in solid-phase than the *p*-nitrophenyl and the pentachlorophenyl (Kupryszewski and Kaczmarek, 1961) esters, although the latter reacts considerably faster in solution. Bodanszky and Bath (1969) commented that relative reactivities measured in solution do not necessarily form a reliable guideline for choosing suitably activated derivatives for use in solid-phase synthesis, a conclusion recently confirmed by Dutta and Morley (1971).

Table XIV—Comparison of Active Ester Reactions in Solid-Phase and in Solution Synthesis ^a

Nucleophile	Half-reaction times (minutes) of active esters of Boc-leucine			
	<i>o</i> -Nitrophenyl ester ONo	<i>m</i> -Nitrophenyl ester ONm	<i>p</i> -Nitrophenyl ester ONp	Pentachlorophenyl ester OPcp
Benzylamine	3	120	15	1
Gly-OBu ^t	12	500	60	4
Val-OBu ^t	90	10,000	840	60
Gly-Resin	150	—	840	1100
Val-Resin	600	—	2400	1800

^a From Bodanszky and Bath, 1969. The reactions were carried out in ethyl acetate at room temperature with 0.02 *M* concentration of the reactants. Rates were measured by the UV absorption of the liberated phenols.

Efforts to improve the effectiveness of *p*-nitrophenyl ester condensations prompted Beyerman *et al.* (1967b, 1968a) and Klostermeyer *et al.* (1967) to study the addition of 1,2,4-triazole which is known to accelerate the reaction (Beyerman and Maassen van den Brink, 1963). Satisfactory coupling yields were obtained in a solid-phase synthesis of a protected oxytocin nonapeptide (Beyerman *et al.*, 1967b, 1968a). However, Ragnarsson *et al.* (1970b) reported that 1,2,4-triazole did not effect any acceleration in couplings of Boc-phenylalanine *p*-nitrophenyl, pentachlorophenyl, *N*-hydroxysuccinimide, and cyanomethyl (Schwyzer *et al.*, 1955) esters with resin-bound alanine. Repeated treatment in *p*-nitrophenyl ester couplings was used by Li and Yamashiro (1970), and imidazole was added in the second reactions. Such "catalyzed" couplings are probably, more or less, azolide rather than active ester condensations.

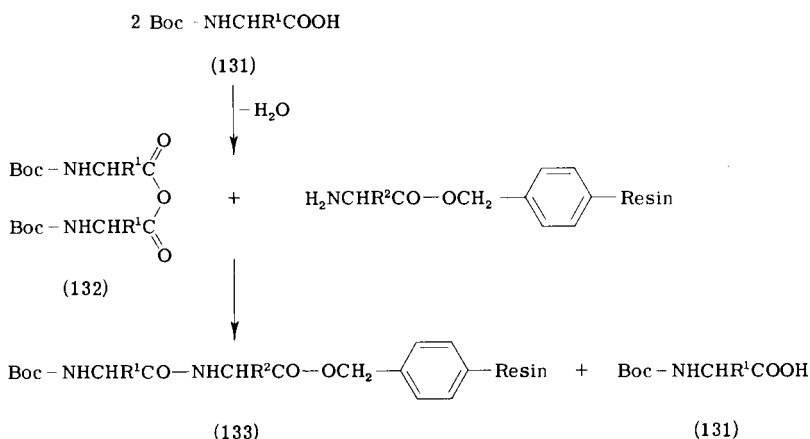
In conclusion, it appears that although being very useful for certain special applications in solid-phase synthesis to date, e.g., for incorporating asparagine and glutamine, active ester couplings are generally slower and less complete than dicyclohexylcarbodiimide couplings.

3. Other Methods and Reagents

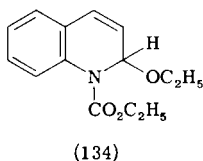
Only a few methods other than those reviewed by Merrifield (1969a, Table V) have since been used in exploratory studies. Carbonyldiimidazole was found by Losse and Klengel (1971) to give sometimes more complete peptide bond formation than dicyclohexylcarbodiimide. The oxidation-reduction condensation of Mukaiyama *et al.* (1970) (see Section VI,B,3) using triphenylphosphine and dipyrityl-2,2'-disulfide in methylene chloride afforded a dipeptide (Leu-Gly) in close to quantitative yield. *N*-Carboxyanhydrides were used to prepare various polyamino acids on the resin (Ingwall *et al.*, 1968; Epand and Scheraga, 1968; Ostroy *et al.*, 1970). The mixed anhydride method (Krumdieck and Baugh, 1969; Baugh *et al.*, 1970a), Woodward's reagent (Khosla *et al.*, 1968), the *o*-phenylene chlorophosphate method (Rothe *et al.*, 1968), and the azide method (Visser and Kerling, 1970; Felix and Merrifield, 1970) have found applications. Direct comparisons of the efficiency of several methods of peptide bond formation in the solid-phase procedure using identical model peptides have been made by Weygand and Ragnarsson (1966), Klostermeyer *et al.* (1967), Baumert and Jakubke (1973), and Ragnarsson *et al.* (1971).

The use of symmetrical anhydrides (132) of *N*-protected amino acids (or peptides), which was first studied by Weygand and Ragnarsson (1966), has been investigated recently in more detail by Wieland and collaborators (Wieland *et al.*, 1971b; Birr *et al.*, 1973). The advantages of this method (131→133) compared to the standard dicyclohexylcarbodiimide procedure

are: (a) the absence of the poorly soluble by-product dicyclohexylurea and, more importantly, (b) the recovery of up to 80% of the excess of *N*-protected amino acid (or peptide) (131). This permits the use of very large excess of acylating component (132) to push the reaction to completion, although in the experimental examples given, the commonly applied ratios of three- to four-fold excesses were applied. A new preparation of symmetrical anhydrides (132) was developed in which sodium salts of *N*-protected amino acids were treated at -40° to -15° with phosgene in tetrahydrofuran; other procedures for the preparation of symmetrical anhydrides (133) have employed dicyclohexylcarbodiimide (Muramatsu and Hagitani, 1959; Weygand *et al.*, 1967) or inamines (Viehe *et al.*, 1964; van Mourik *et al.*, 1965). Solid-phase syntheses of antamanide by the symmetrical anhydride method (Wieland *et al.*, 1971b) gave 60% of linear decapeptide precursor when crystalline anhydrides were used and 96% (!) of homogeneous decapeptide when the anhydrides were added *in situ* (in solution, immediately after preparation without isolation) to the peptide-resin. From the filtrates the excess Boc-amino acids were recovered (up to 80%) after hydrolysis of remaining anhydride with aqueous bicarbonate. This "economic" coupling method appears to have considerable promise.



A novel reagent for peptide bond formation *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (134) (Belleau and Malek, 1968) has been applied to solid-phase synthesis by Windridge and Jorgensen (1971) and by Sipos and Gaston (1971).



Jakubke and Baumert (1973) compared the coupling effectiveness of seven procedures. Dicyclohexylcarbodiimide in methylene chloride proved to be considerably superior to both the mixed carboxylic carbonic anhydride method [using isobutyl chloroformate in tetrahydrofuran-methylene chloride (2:3)] and the Woodward procedure [using *N*-ethyl-5-phenylisoxazolium-3'-sulfonate in acetonitrile-dimethylformamide (1:2)]. Among the active esters which were studied (in methylene chloride), the 5-chloroquinolyl-(8) esters (Jakubke *et al.*, 1967) reacted at a faster rate than the *p*-nitrophenyl, selenophenyl (Jakubke, 1964), and *N*-hydroxyurethane esters (Jeschkeit, 1969).

4. Fragment Condensation

The coupling of protected peptide fragments to the growing resin-bound peptide chain appears to be an ideal strategy (Anfinson, 1968) for syntheses of large polypeptides or of proteins. By such a procedure the gradual buildup of microheterogeneous contamination, consisting of countless closely related failure sequences, through the cumulative effect of incomplete reaction at each amino acid incorporation, would be avoided. Failure sequences arising during solid-phase fragment condensation will differ from the desired product by the lack of relatively large parts of the molecule. Although it is certain that the coupling of fragments with resin-bound peptides will be even less complete than that of Boc-amino acids, this could be more than compensated by obvious advantages: (a) the degree of heterogeneity (or the amount of contaminating incomplete parts) of the final product can be quantitatively evaluated by presently available analytic methods, and (b) the purification of the target peptide should be facilitated and experimentally feasible with existing techniques. Within limits set by considerations of coupling efficiency and of minimal racemization, a strategic subdivision of a given target sequence would be the alternating of acidic fragments (containing more Glu and Asp than Lys and Arg) with basic fragments. The purification of the final product would thus be facilitated, e.g., by ion exchange or electrophoretic techniques. Similarly, the analytic monitoring of purification and the assessment of homogeneity could be made more accurate through strategic use of certain infrequently occurring or single amino acid residues as markers. Strategically located analytic markers have been named "diagnostic" amino acid residues (Hofmann, 1973; Beachman *et al.*, 1971). For large polypeptides or proteins containing no single residues, radioactively labeled amino acids could be used.*

Exploratory investigations of solid-phase fragment condensation (Anfin-

* This strategy has been employed to determine the degree of polymerization of sequence polymers (prepared by solution synthesis) with the aid of a [¹⁴C]-labeled C-terminal (Johnson, 1971).

sen, 1968; Omenn and Anfinsen, 1968; Weygand, 1968) showed encouraging results. N-Protected tri- or tetrapeptides were condensed to resin-bound di- or pentapeptides using *N*-ethyl-5-phenylisoxazolium-3'-sulfonate (Woodward and Olofson, 1961) or *N*-hydroxysuccinimide—dicyclohexylcarbodiimide (Weygand *et al.*, 1966). Kusch (1966a,b) used units obtained by condensing hexamethylenediamine and adipic acid to prepare nylon oligomers; Rothe *et al.* (1968) prepared sequence polymers (collagen models) by coupling one to six Boc-Pro-Gly-Gly to Pro-Gly-Gly-Resin with the use of *o*-phenylene chlorophosphite in diethyl phosphite as a solvent; and Sakakibara *et al.* (1968) synthesized (Pro-Pro-Gly)_{*n*} (*n* = 9, 19), similarly, but with the aid of dicyclohexylcarbodiimide. Didepsipeptide units (Gisin *et al.*, 1969; Gisin and Merrifield, 1970; Losse and Klengel, 1971) and tetradepsipeptide fragments (Losse and Klengel, 1971) have been used in syntheses of valinomycin. Z-Gly-Gly was condensed to a resin-bound hexapeptide to prepare an angiotensin analog (Jorgensen *et al.*, 1969, 1970c), and Boc-Leu-Ala was successfully coupled to an amino acyl resin (Esko and Karlsson, 1970).

Cobrotoxin-(47–52) prepared by fragment condensation (3 + 3-resin) using dicyclohexylcarbodiimide with or without *N*-hydroxysuccinimide was found by Noda *et al.* (1970b) to be more homogeneous than a product prepared by the usual stepwise solid-phase synthesis. The largest fragments coupled to date to peptide-resins are hexa- and octapeptides. Omenn and Anfinsen, as early as 1968, condensed Boc-Met-Thr-Phe-Arg(NO₂)-Leu-Leu-Val-OH to Asp(OBzl)-Thr-Pro-Glu(OBzl)-Thr-Lys(Tfa)-His-Pro-Lys(Tfa)-resin by dicyclohexylcarbodiimide. Recently Yajima and collaborators (1970c) described the synthesis of ACTH-(5–14) by condensing fragment 5–10 to the resin-bound tetrapeptide (sequence 11–14) with the use of dicyclohexylcarbodiimide. A tridecapeptide, [Cit¹⁰, Leu¹³]-ribonuclease A-(1–13), was obtained from treatment of a pentapeptide-resin (9–13) with an octapeptide azide (Visser and Kerling, 1970). Fragment condensation has also been successful on a macroreticular support (Sano *et al.*, 1971).

As Sheppard (1973) observed, further evidence will be necessary before it is known whether sufficiently high yields in fragment condensation reactions onto polymers are readily attainable and whether any lowering of yield is counterbalanced by simplification of the purification problems. The viability of the approach will also need to be assessed in terms of the several-fold excesses of each of the acylating reactants which have customarily been used in solid-phase synthesis in the past. The excess of carboxyl component is not recoverable in unaltered form after dicyclohexylcarbodiimide, azide, or mixed anhydride mediated condensations. However, excesses of active

esters of protected peptides could very likely be reclaimed intact and used again.

Solid-phase fragment condensation could become potentially useful for another purpose: to avoid side-reactions or failures during coupling of protected amino acids in certain particularly unfavorable sequences. For example, the observed racemization during the incorporation of Boc-*N*^{im}-benzylhistidine into angiotensin analogs (Jorgensen *et al.*, 1970a) could, perhaps, be bypassed by incorporating the fragment Boc-His(Bzl)-Pro-OH. A fragment condensation was studied (Meienhofer and Trzeciak, 1970) in an effort to bypass difficulties observed with the incorporation of the glutamine residue in syntheses of lysine-vasopressin. Treatment of Cys(Bzl)-Pro-Lys(Tos)-Gly-Resin with Boc-Phe-Gln-Asn-ONp gave 70% coupling; and 80% (after recrystallization) of the applied excess of Boc-tripeptide nitrophenyl ester was recovered. Surprisingly, all attempts to condense Boc-Tyr(Bzl) with the ensuing heptapeptide-resin met with failure.* Presumably the peptide obtained through the fragment condensation is somehow folded or interacts with the solid-support so that the N-terminal amino group becomes completely inaccessible.

Obviously, fragment condensation also lends itself to combining solid-phase and solution synthesis in reverse order, i.e., preparation of fragments by solid-phase synthesis and coupling in solution to form a larger peptide. A synthesis of angiotensin was carried out in this manner by Wang and Merrifield (1971).

E. REMOVAL OF THE SOLID SUPPORT

After the peptide chain has been assembled, the covalent linkage to the solid support is cleaved and the solid is then removed. The standard benzyl ester linkage to the cross-linked polystyrene resin is cleaved in the majority of syntheses by acid, using either hydrogen bromide in trifluoroacetic acid or hydrogen fluoride. Unfortunately, these procedures cause side reactions and decomposition of products which become more extensive when large synthetic peptide or protein preparations are cleaved from the solid support. Nevertheless, acidolysis is still overall the most useful cleavage method. Alternative procedures are ammonolysis producing C-terminal amides, hydrazinolysis to give hydrazides, transesterification leading to alkyl esters and alkaline hydrolysis. The deleterious effects of acidolysis have prompted searches for modified peptide-resin linkages that will allow cleavage under

* After removal of the solid support, isolated heptapeptide coupled readily in solution with Boc-Tyr(Bzl). Incorporation of Boc-Tyr(Bzl) into heptapeptide resin prepared by standard incremental chain elongation also proceeded normally.

much milder conditions. These modified supports and peptide attachments have been discussed in Sections III,A and B. The respective cleavage procedures will be described below.

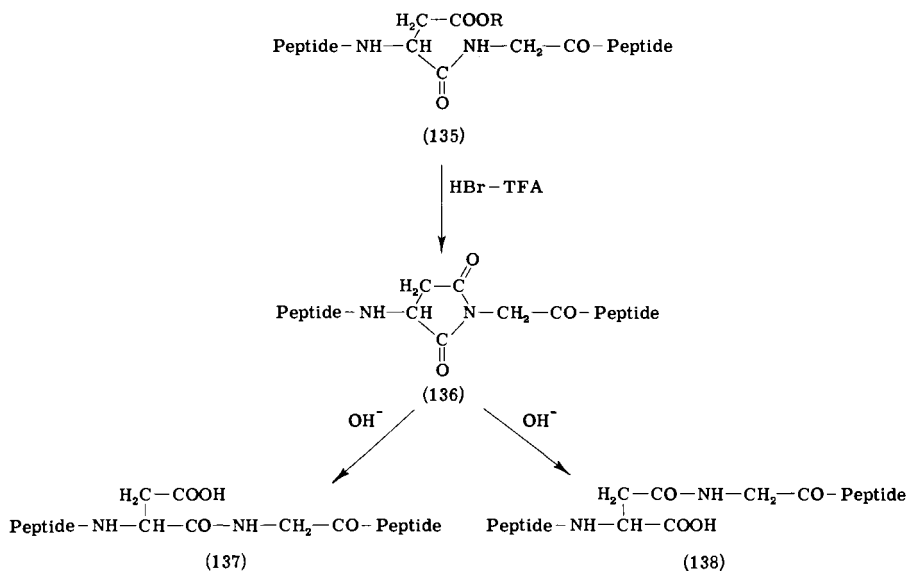
1. *Cleavage of the Standard Benzyl Ester Linkage*

a. Acidolytic Cleavage. Both the hydrogen bromide in trifluoroacetic acid and the anhydrous hydrogen fluoride procedures are simple and do not provide for a great deal of variation except for the duration of the treatment and the temperature. Both variables depend to a large extent on the individual peptide, and optimal conditions have to be determined for each case separately.

i. Cleavage by Hydrogen Bromide. Hydrogen bromide in acetic acid was first used by Ben-Ishai and Berger (1952) to cleave the benzyloxycarbonyl group. Trifluoroacetic acid was introduced as a solvent (Guttmann and Boissonnas, 1958) to avoid acetylation of the serine or threonine hydroxyls. The effectiveness of the procedure in solid-phase synthesis for the cleavage of the benzyl ester linkage between the peptide and the resin has been reviewed by Merrifield (1969a). A suitable apparatus and experimental details are described by Stewart and Young (1969). Care should be taken to assure the exclusion of moisture during the acidolysis. Hydrogen bromide in a 1:1 mixture of trifluoroacetic acid and methylene chloride has also been used (Merrifield, 1967; Li and Hemmami, 1972). A kinetic study (Merrifield, 1967) of the rate of cleavage of angiotensinyl-bradykinin from the solid support by hydrogen bromide in trifluoroacetic acid showed 57% cleavage after 5 minutes, 64% after 10 minutes, 74% after 30 minutes, and 85% after 1 hour.

A number of provisions are required to minimize undesired side reactions. Side reactions known from solution synthesis include *S*-benzylsulfonium formation of methionine (Guttmann and Boissonnas, 1959; Albertson and McKay, 1953), and of cysteine derivatives which can be avoided or minimized by adding methyl ethyl sulfide (Merrifield, 1964c) or methionine (Marglin and Merrifield, 1966) to the reaction mixture. Addition of anisole or resorcinol serves to trap any *tert*-butyl cation (Kessler and Iselin, 1966). Side reactions due to the presence of bromine in hydrogen bromide, e.g. bromination of tyrosine residues (Weygand and Steglich, 1959), are avoided by passing the hydrogen bromide gas through a solution of resorcinol or anisole in trifluoroacetic acid prior to the introduction into the reaction mixture (Marglin and Merrifield, 1966; Anfinsen, 1968), or by adding phenol to the reaction mixture. Peptides containing tryptophan residues which are very sensitive to oxidation in acidic media also can be successfully removed from solid supports by acidolysis especially when reducing agents, such as

β -Mercaptoethanol (Blake and Li, 1968; Marshall, 1968), are added to the reaction mixture. A side reaction originating from aspartyl β -alkyl ester residues (135), formation of succinimidoyl (cyclic diacylimide) derivatives, has been observed by Ondetti and collaborators (1968a) and probably is confined to peptides containing Asp-Gly and possibly Asp-Ser sequences. The succinimido derivative (136) opens readily under alkaline conditions; but the ensuing mixture might consist predominantly of the undesired β isomer (138). It is, therefore, important to consider the possible occurrence of this side reaction in planning a solid-phase synthesis when the peptide contains aspartyl sequences (135, 137) (see, e.g., Marshall and Merrifield, 1965). Thus, Jorgensen *et al.* (1971a) detected succinimido-side product formation in a solid-phase synthesis of [2-glycine]-angiotensin II, which contained the Asp(OBzl)-Gly sequence.



The most aggravating disadvantages of the hydrogen bromide in trifluoroacetic acid reaction, however, are (a) the overall destructive effects on the synthetic products, which have been considerable with some larger peptides, and which demand that reaction times be kept as short as possible; and (b) incomplete cleavages which call for prolonged treatments. This dilemma is reflected in the wide variations of time and temperature used in the cleavage reaction, e.g., 5 minutes at 25° (Marshall and Merrifield, 1965), 2 hours at 2° (Hancock *et al.*, 1971; Marshall *et al.*, 1973), or 2 hours at 25° (Ohno *et al.*, 1969).

Anhydrous trifluoroacetic acid alone at elevated temperature (75°) and for a prolonged period of time (18 hours) has also been used in one instance (Merrifield, 1967) to cleave angiotensinyl-bradykinin from the solid support in 98% yield.

ii. *Cleavage by Anhydrous Hydrogen Fluoride.* Lenard and Robinson (1967) used the facile benzyl ester cleavage by anhydrous hydrogen fluoride (Sakakibara and Shimonishi, 1965) for the cleavage of the peptide to resin linkage. The hydrogen fluoride procedure, which recently has been reviewed by Sakakibara (1971), seemed to give somewhat higher yields of purer products than the hydrogen bromide in trifluoroacetic acid cleavage in comparative studies (e.g., Gutte and Merrifield, 1971). Less tryptophan degradation was observed (Marshall, 1968). Distillation of hydrogen fluoride from CoF_3 eliminates traces of moisture. One hour reaction at 0° is usually used, but 30 minutes at 0° also has been found adequate (Scotchler *et al.*, 1970). Anisole is routinely added to the cleavage reaction as a scavenger of nitronium, *tert*-butyl and benzyl cations which would otherwise react with tyrosine, methionine, and S-alkyl cysteine derivatives. Methionine has been used in the sulfoxide form (Gutte and Merrifield, 1971) as a protection against sulfonium ion formation and against cleavage that could potentially occur at this residue (Lenard *et al.*, 1964; Lenard, 1969). In the removal reaction of a related peptide, however, unmodified methionine was used without noticeable difference.

Special equipment is required. An inexpensive apparatus constructed from readily available polyethylene or polypropylene tubing, connectors, valves, and screwcap bottles has been described (Pourchot and Johnson, 1969). A more sophisticated apparatus (Sakakibara *et al.*, 1967a,b) made of Kel-F (commercially available from Peninsula Laboratories, 1105 Laurel Street, San Carlos, California 94070), (Fig. 15) allows the safe handling of larger volumes of hydrogen fluoride,* even at elevated temperatures. Experimental details are given by Sakakibara (1971) and by Stewart and Young (1969).

The advantage of the HF procedure is the simultaneous cleavage of most side-chain protecting groups used in solid-phase synthesis. With appropriate planning no additional operation would be required for complete deprotection because HF-removable side-chain protecting groups are known for every amino acid (see Table XXVII, p. 231). A mixture of hydrogen fluoride and trifluoroacetic acid has also been used for cleavage of the pep-

* Liquid hydrogen fluoride is strongly toxic and extremely hazardous. On contact with skin it causes severe burns that penetrate deeply. Treatment of HF burns is described by Sakakibara (1971). Subcutaneous calcium gluconate (Upjohn) infiltration must be used.

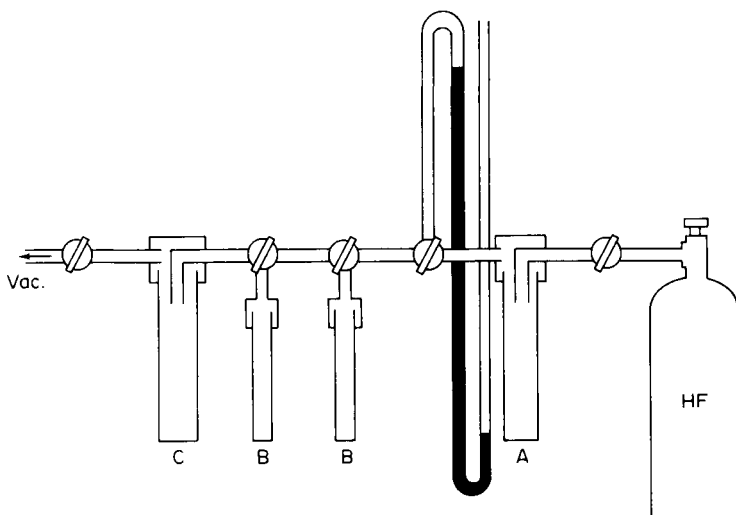


FIG. 15. All Kel-F vacuum system for general hydrogen fluoride reactions. A, Hydrogen fluoride reservoir; B, reaction vessel; C, hydrogen fluoride trap. From Sakakibara *et al.* (1967a,b).

tide to resin linkage (Gutte and Merrifield, 1971), but side-product formation was evidently greater than with HF alone.

b. Ammonolysis. Ammonolysis of the peptide from the solid support was first carried out by Bodanszky and Sheehan (1964). The procedure is most convenient for syntheses of peptides with a C-terminal amide, for example, thyrotropin releasing hormone, oxytocin and vasopressin, α -melanotropin, gastrin, secretin, or certain kinins, such as eleodisin and physalaemin. Yields usually are good and sometimes close to quantitative as in preparations of Z-Pro-Lys(Tos)-Gly-NH₂ (Meienhofer and Sano, 1968), or of a protected nonapeptide of [Thr⁴]-oxytocin (Manning *et al.*, 1970). No racemization could be detected gas chromatographically after ammonolysis of a peptide with an optically active C-terminal amino acid (Bayer *et al.*, 1971a). The conditions for the ammonolytic reaction have been adopted from solution synthesis (e.g., du Vigneaud *et al.*, 1953; Ressler and du Vigneaud, 1954) and involve treatment of the peptide-resin in a sealed flask for 1–3 days at room temperature with methanol, ethanol, or alcohol–dimethylformamide mixtures that have been presaturated with ammonia at 0°. Beyerman *et al.*, (1968b) observed that the reaction proceeds, in part, through base-catalyzed transesterification to give the ester corresponding to the alcohol used.

Z-Pro-Leu-Gly-OMe was isolated after a 1-hour ammonolysis; in our laboratory up to 40% Z-Pro-Lys(Tos)-Gly-OEt along with 60% of the respective amide, was obtained after 6 hours (Meienhofer and Trzeciak, 1970). It is possible that peptide-resin ammonolysis in alcoholic solutions occurs entirely via transesterification. Ammonolytic removal of a hexapeptide with a bulky C-terminal residue, Boc-Leu-Leu-Gln-Gly-Leu-Val-resin, gave the methyl ester only (Bodanszky and Sheehan, 1966). It is interesting in this context that treatment with liquid ammonia resulted in much lower yields of Z-Pro-Leu-Gly-NH₂ (Beyerman *et al.*, 1967b) and of a protected nonapeptide with oxytocin sequence (Beyerman *et al.*, 1968a) than those obtained from alcohol saturated with ammonia.

A novel procedure for ammonolysis was devised by Bayer *et al.* (1971a) which is reported to give better yields. The peptide resin is kept in a 1:1 mixture of dimethylformamide and liquid ammonia in a sealed tube for 2 days at 0° and for 8 days at room temperature.

Cleavage of peptides from the solid support by substituted amines, *aminolysis*, has been used also. *N*-Benzyloxycarbonyl-S,S'-dibenzoyloxycinoic acid methylamide and hydroxamide (Beyerman and Maassen van den Brink-Zimmermannová, 1968) have thus been prepared, as well as deamino-oxytocinoic acid methylamide and deamino-oxytocinoic acid dimethylamide (Takashima *et al.*, 1969).

c. Hydrazinolysis. Hydrazides of protected peptides can be readily prepared by direct hydrazinolysis of resin-bound acyl peptides. This was first examined by Kessler and Iselin (1966) and by Ohno and Anfinsen (1967). Side-chain protecting groups labile to hydrazine must necessarily be avoided, e.g., ω -esters of aspartic and glutamic acid residues, *N*-formyl, *N*-phthalyl, or *N*-trifluoroacetyl groups. The amide functions of asparagine and glutamine might also be attacked (MacLaren *et al.*, 1958). Anhydrous hydrazine or hydrazine hydrate (20–200 equivalents) in methanol, ethanol, or dimethylformamide have been used (Table XV). Visser *et al.* (1968) observed that dimethylformamide is the superior solvent for peptide-resin hydrazinolysis; a methanolic solution of hydrazine hydrate resulted in lower yields and impure products, probably because methanol is not a good swelling solvent for the polymer. Yields obtained by hydrazinolysis have been very good (over 70%); the lower yields obtained by Kessler and Iselin (1966) and by Pettit and Jones (1971) could be due to a too short reaction period or to incomplete extraction of the cleaved peptide from the resin, respectively. Repeated extraction had to be used by Beyerman and Maassen van den Brink-Zimmermannová (1968). In spite of its effectiveness, peptide-resin hydrazinolysis has not yet found many applications (Table XV). The procedure provides convenient intermediates for peptide fragment con-

Table XV.—Protected Peptide Hydrazides obtained by Hydrazinolysis of Peptides bound to Polystyrene-2% Divinylbenzene through Benzylester Linkage

Protected peptide hydrazides	Equiv. N ₂ H ₄	Yield (%)	Solvent	Time (hours) at 20°C	Reference
Z-Phe-Lys(Boc)-Gly-N ₂ H ₄ cryst.	220	30	EtOH-N ₂ H ₄ · H ₂ O 4:1	6	Kessler and Iselin (1966)
Boc-Leu-Ala-Tyr-Ile-Tyr-Ala-N ₂ H ₄ cryst.	30	75	DMF-N ₂ H ₄ 16:1	48	Ohno and Anfinsen (1967)
Dns-Pro-N ₂ H ₄ (n = 1-12)	200	—	EtOH-N ₂ H ₄ 5:1	20	Stryer and Haugland (1967)
Boc-Leu-Ala-Tyr(Bzl)-Ile-Tyr (Bzl)-Ala-N ₂ H ₄	30	—	MeOH-H ₂ O	36	Anfinsen (1968)
Boc-Thr-Ala-Ala-N ₂ H ₄	57	82	DMF-N ₂ H ₄ · H ₂ O 12.5:1	48	Visser <i>et al.</i> (1968)
Boc-Lys(Boc)-Glu(OBu')-Thr- Ala-Ala-Ala-Lys(Z)-Phe-N ₂ H ₄	20	70	DMF-N ₂ H ₄ · H ₂ O 17:1	72	Visser <i>et al.</i> (1968)
Z-Cys(Bzl)-Tyr-Ile-Gln-Asn-Cys (Bzl)-Pro-Leu-Gly-N ₂ H ₄ cryst.	1900	74	MeOH-N ₂ H ₄ · H ₂ O 1:1	2	Beyerman and Maassen van den Brink-Zimmermannová (1968)
Z-Thr-Ala-Leu-Leu-Gly-N ₂ H ₄	60	17	DMF-N ₂ H ₄ 3.5:1	67	Pettit and Jones (1971)
Meoz-D-Phe-Pro-Val-Orn(Z · NO ₂)- Leu-D-Phe-Pro-Val-Orn(Z · NO ₂)- Leu-N ₂ H ₄ cryst.	10	97	DMF-N ₂ H ₄ · H ₂ O	48	Ohno <i>et al.</i> (1971)

* Peptide hydrazides obtained from other solid supports are not included.

densation and allows combination of solid-phase and solution synthesis. The most impressive application of hydrazinolysis, and of solid-phase synthesis per se is the preparation by Izumiya and collaborators of the crystalline decapeptide derivative, Meoz-D-Phe-Pro-Val-Orn($Z\cdot NO_2$)-Leu-D-Phe-Pro-Val-Orn($Z\cdot NO_2$)-Leu-NHNH₂, in close to quantitative overall yield (based on the Meoz-leucine-resin) during an improved synthesis of gramicidin S (Ohno *et al.*, 1971).

d. Alcoholysis. Alcoholysis of resin-bound peptides with the aid of a strong ion exchange resin (Bio-Rad AG1-X8, hydroxide form) was shown to give 50–70% yields of peptide esters (Halpern *et al.*, 1968; Pereira *et al.*, 1969b) after 2 hours treatment at room temperature. Presumably, alkoxide ions mediate these conversions (Pereira *et al.*, 1969a). Preservation of optical purity (<1% of diastereoisomers) was demonstrated by a gas chromatographic test (Westley and Halpern, 1968).

The observation of intermediate transesterification during peptide-resin ammonolysis prompted Beyerman and collaborators (1968b, 1971a) to study tertiary base catalyzed transesterification as a means to remove the solid support under mild conditions, thereby affording protected peptide esters. Z-Pro-Leu-Gly-OEt was isolated in 55% yield after a 17-hour treatment at room temperature of the peptide-resin with 1 *N* ethanolic triethylamine; the corresponding methyl ester was obtained in 90% yield after a 4-hour conversion by *N*-methylpiperidine in methanol. *N*-Benzyloxycarbonyl-*S,S'*-dibenzyl-*O*-benzyloxytocinoic acid methyl esters was produced in 70% yield.

o-Nitrophenylsulfenyl-dipeptide resins have been used to study the kinetics of alcoholysis (Beyerman, 1972) by measuring the extinction at $\lambda = 368$ or 378 nm of the liberated Nps-peptide ester in the solution. Linearity of release with time was observed up to about 80% alcoholysis, and the half-reaction time was approximately 7 minutes. Roepstorff *et al.* (1970) removed acylpeptides from the resin as methyl esters by treatment with methanol–10% triethylamine for 24 hours at ambient temperature. Boc-Val-Pro-Pro-Phe-Phe-OMe was obtained from a resin by transesterification using ethyl diisopropylamine in methanol (Wieland *et al.*, 1971a). So far, the largest peptide that has been removed from the resin by alcoholysis was a protected intermediate in a synthesis of the tridecapeptide [5-glutamine]- α -melanotopin, Ac-Ser(Bzl)-Tyr(Bzl)-Ser(Bzl)-Met-Gln-His(Bzl)-Phe-Arg(Tos)-Trp-Gly-Lys(Tos)-Pro-Val-OMe, which was obtained after a 22-hour treatment of the peptide-resin at 42° with a mixture of methanol–dimethylformamide–triethylamine (25:25:11) (Blake *et al.*, 1970).

e. Alkaline Saponification. Removal of peptides from the standard resin support by alkaline saponification [e.g., by 0.2 *N* NaOH in aqueous ethanol (Marshall and Merrifield, 1965)] is not a generally applicable procedure. β -Benzylaspartyl residue rearrangement produces mixtures of α - and β -aspartyl peptides containing predominantly the β isomer. Early work has been reviewed by Merrifield (1969a). Racemization was found to occur in a related (modified) resin support (Bayer *et al.*, 1971a). Recently, nitrated polystyrene-2% divinylbenzene resin was cleaved from a di- and a tripeptide by 0.2 *N* NaOH in 90% ethanol (Arai *et al.*, 1970). Several polymyxin-related peptides, up to heptapeptides were released from the standard resin by two successive 30-minute treatments with 2 *N* NaOH in methanol (Morozova and Zevail, 1970a,b; Zevail and Morozova, 1970, 1971). The C-terminal peptides of bovine insulin B-chain, Z-Thr-Pro-Lys(Tos)-Ala and Phe-Phe-Tyr-Thr-Pro-Lys(Tos)-Ala, were also obtained after removal of the solid support by alkaline saponification (Vdovina *et al.*, 1971).

2. Cleavage of Other Types of Peptide to Resin Linkage

Main incentives for the development of alternative types of linkage of peptides to solid supports, as described earlier (Section III,B,2), were attempts to find milder reaction conditions for the removal of the support from the completed peptide and to obtain higher yields of products. The urgent need for alternative removal procedures that would be considerably milder than the presently used acidolytic conditions, required for cleavage of the benzyl ester bond, has received recently strong and repeated emphasis. It has been pointed out in several reports on solid-phase syntheses of large polypeptides and proteins that large losses of activity occurred when the respective natural compound was subjected to the same acid treatment as required for the removal of the solid support. Such activity losses were observed with bovine parathyroid hormone (Potts *et al.*, 1971); *Escherichia coli* acyl carrier protein (Hancock *et al.*, 1971; Marshall *et al.*, 1973); and human growth hormone (Li and Yamashiro, 1970). It is a particularly distressing aspect that in the penultimate stage of a synthesis considerable portions of a peptide should be degraded, which has just been assembled through a multitude of stages. However, it should "be pointed out that there is little merit in expressing biological activity as a fraction of that exhibited by the natural product after it has been submitted to similar damaging treatments" (Sheppard, 1973).

For peptides attached to the resin by bonds other than the standard benzyl ester, a wide range of cleavage conditions are available. These reactions

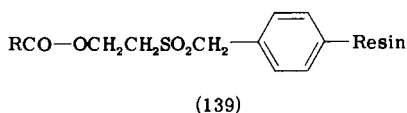
include specific enzymic cleavage, mild acidolytic scission, various base treatments, and activation of safety-catch bonds followed by cleavage.

The hexapeptide Leu-Ala-Lys-Val-Ala-Tyr was released from a polypeptide support (61) by specific cleavage of its peptide linkage (72) through treatment with chymotrypsin (Anfinsen, 1968). Thermolysin digestion served to cleave other peptide to polymer bonds.

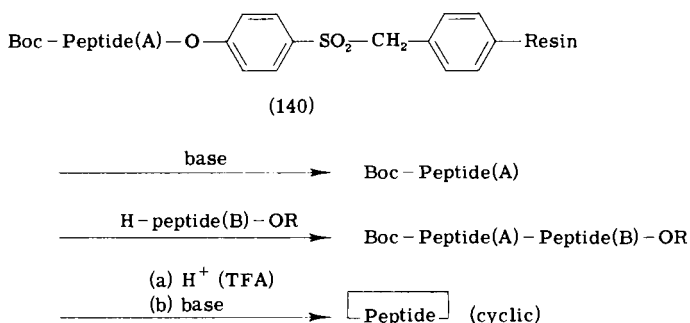
Very mild acid treatment is used to cleave linkages such as the benzhydryl ester bond, the *tert*-alkyl ester bond, and the *tert*-alkyloxycarbonylhydrazide bond. The benzhydryl ester linkage (70) is severed by treatment with 5% (to 50%) trifluoroacetic acid in chloroform (5–30 minutes), with 0.03 *N* boron trifluoride in acetic acid–chloroform (7:35), or with 0.4 *N* hydrogen chloride in tetrahydrofuran–chloroform (2:13) (Southard *et al.*, 1969, 1971c). Both the *tert*-alkyl ester linkage (71) and the *tert*-alkyloxycarbonylhydrazide linkage (80) have been cleaved by treatment with 50% trifluoroacetic acid in methylene chloride for 30 minutes at room temperature (Wang and Merrifield, 1969a, 1971). Stronger acid (hydrogen bromide in dioxane, or in trifluoroacetic acid for 15–20 minutes) has been used to cleave the benzylurethane linkage (90) (Meienhofer and Trzeciak, 1971). These conditions are still somewhat milder than those commonly applied for the benzyl ester cleavage. Cleavage of benzhydrylamide bonds (81) to produce peptide amides requires treatment with hydrogen fluoride (Pietta and Marshall, 1970; Pietta *et al.*, 1973).

Various base treatments have been used in cleaving other types of linkages. Reaction with sodium thiophenoxide in dimethylformamide for 5 hours at room temperature served to cleave the phenacyl ester linkage (68) (Weygand, 1968; Mizoguchi *et al.*, 1969, 1970). Alternatively, 0.5 *N* sodium hydroxide in dioxane has been used, or methanolic ammonia to give peptide amides, methanolic hydrazine to give hydrazides, or triethylamine containing methanol to give peptide methyl esters. Very mild alkaline saponification (approximately twofold excess of 0.05 *N* aqueous sodium hydroxide in dioxane-ethanol, 1:1) has been used by Tilak and Hollinden (1968a,b) in cleaving alkyl ester linkages between peptides and supports of types (55) and (56). Alkyl ester linkages between peptides and the 6-hydroxy-*n*-hexyl resin (54) were severed (Bayer *et al.*, 1971a) by either 4 *N* sodium hydroxide in dioxane (1:1) or by a 1:1 mixture of dimethylformamide and liquid ammonia (2 days at 0° and 8 days at room temperature) to give peptide amides, even with those possessing C-terminal valine [see Bodanszky and Sheehan (1966) for difficulties observed in ammonolysis of peptides with sterically hindered C-terminal amino acid residues]. Peptide alkyl ester bonds to Sephadex supports were cleaved by ammonolysis or hydrazinolysis in methanol (Vlasov and Bilibin, 1969). The phenyl ester linkage

(79) has been severed by treatment with 0.14 *N* sodium hydroxide in a 1:1 mixture of ethanol and dimethylformamide (2 hours at room temperature) (Inukai *et al.*, 1968). Amides and hydrazides were obtained after treatment with 25% ammonia in methanol or hydrazine hydrate in dimethylformamide, respectively. Blake and Li (1971) observed extensive racemization of the C-terminal valine residue in α -melanotropin when ammonolysis of the phenyl ester linkage to resin 56a was carried out at room temperature. The reaction had to be performed at -20° to avoid this problem. The ethylsulfonylbenzyl ester linkage (139) was cleaved by sodium methoxide in methanol (Tesser and Ellenbroek, 1967).

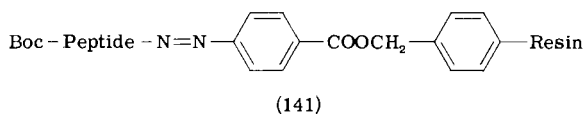


The removal of peptides from solid supports that are linked through safety catch bonds involves two successive reactions, the activation of the bond (as discussed in Section III,B,2) and the actual cleavage. The methylsulfonylphenyl ester intermediate (83) obtained by oxidative activation of the 4-methylthiophenyl ester linkage (73) is readily cleaved by mild base treatment using, e.g., 2% triethylamine in dimethylformamide (18 hours at room temperature). The cleavage can be combined with simultaneous peptide bond formation, thereby elongating the chain at the C-terminal end. To achieve this, the oxidized peptide-resin (140) is treated with peptide esters. Cyclization can also be carried out when the base treatment of (140) is preceded by removal of the N-protecting group.



By analogous procedures peptides, elongated peptides, or cyclic peptides should be obtainable through the acylazo intermediate (141) derived from

the 4-carboxyphenylhydrazide linkage (88) by *N*-bromosuccinimide oxidation (Wieland *et al.*, 1970). A linear antamanide precursor decapeptide was



synthesized in excellent yield (87%) using this system; however, cyclization directly on the solid-phase failed to give more than trace amounts of antamanide (Wieland *et al.*, 1970). The methylated acylsulfonamide linkage (89) can be cleaved by treatment with 0.5 *N* sodium hydroxide, with excess of 0.5 *N* ammonia in dioxane, or with 3 equivalents methanolic hydrazine.

Several of these alternative peptide to resin linkages appear to have considerable potential in enhancing the scope of solid-phase synthesis. However, it is difficult to predict at this time which system might prove to be so effective as to find wide application.

F. PURIFICATION AND CHARACTERIZATION

1. General Considerations

Merrifield (1969a) has reiterated the necessity of applying powerful purification procedures to products of solid-phase synthesis. He stated that "even with ideal conditions and very nearly quantitative yields at every step it is too much to expect, and has never been claimed, that pure products will be obtained directly."

The majority of methodological studies with the aid of recently developed analytic procedures (see Section IV,D) have shown that at the present state of development and with the commonly used operations *conditions are far from ideal*. It is essential that this fact be recognized and always kept in mind by those who are using or planning to use the method. There is, on the other hand, little merit in making this point the bone of contention and in eternalizing sterile argumentation. Those who require pure preparations represent-

ing single molecular entities of peptides of, say, larger than pentadecapeptides should not, at present, use solid-phase synthesis. Those who decide to use the method and to benefit from the dramatic time savings offered by it must, however, face the consequences of spending considerable amounts of time, effort, skill, and sophistication in (a) purification of the product, and (b) characterization of purified materials to demonstrate homogeneity. It is necessary to apply the most powerful modern multistage fractionation procedures, preferably, several in sequence, to effect purification. Such procedures include countercurrent distribution, chromatography (comprising partition, ion exchange, thin-layer, molecular sieve, or affinity techniques), high voltage or gel electrophoresis, isoelectric focusing, functional purification, and, last but not least, crystallization. Many of these procedures have been applied to the purification of solid-phase synthetic peptides depending on the availability of instrumentation. The optimal fractionation of very complex mixtures of large synthetic peptides or proteins requires not only the successive use of several different highly effective procedures, but also a great deal of experience on the part of the investigator. Crucial questions for each solid-phase synthesis are: (a) To what degree can the product mixture finally be purified, and (b) Will the attainable degree of purity of the synthetic peptide be sufficient for an unambiguous and conclusive solution of the problem under investigation?

Solid-phase synthesis has created another serious problem with respect to product analysis and to assessment of purity. The analytic methods that have been developed in the past 25 years for the characterization of both proteins and synthetic peptides are just plainly inadequate for solid-phase synthetic peptides. Single components of microheterogeneous mixtures escape individual detection or identification. The degree of complexity of the mixture cannot even be determined. Anfinsen (1968) has proposed a possible approach: "at the moment, I would suggest that only the quantitative analysis of specific enzyme digests—quantitative fingerprints in a sense—can give a true estimation of deviation from homogeneity." This type of fingerprinting has, however, to date never been applied to a solid-phase synthetic product for good reasons: such a test would amount to about half a sequence analysis.

In the following sections purification procedures will be briefly discussed followed by an account of analytic product control. It would exceed the limits of this review to discuss these methods in detail or to list all the uses recorded with synthetic peptides prepared by the solid-phase method. Literature quotation is, therefore, confined to several reviews and some selected original publications. An illustrative example will be described, where a very complex mixture [synthetic ribonuclease A (Gutte and Merrifield, 1971)]

has been purified approximately 10- to 20-fold through several successive stages of chromatography and functional purification.

2. Preparative Fractionation Procedures

By far the most frequently applied purification technique for solid-phase synthetic peptides is ion exchange chromatography. The next most popular procedure is gel filtration followed by countercurrent distribution, and crystallization.

Ion exchange chromatography on Dowex 1 $\times$ 2 has served for the purification, for example, of Ile⁵-Angiotensin II (Fujii and Sakakibara, 1970), and of tobacco mosaic virus protein-(42-46) (Young *et al.*, 1968a) after removal from the solid support by hydrogen fluoride treatment. Dowex 50 was used to purify, e.g., an octapeptide [lysozyme-(64-71): Jollès and Jollès, 1968], thyrotropin releasing factor (Baugh *et al.*, 1970a), and to separate Leu-Ala-Val from Leu-D-Ala-Val (Esko and Karlsson, 1970). Carboxymethyl cellulose was effective, e.g., for purification of α -ACTH-(4-10) (Blake and Li, 1968), of parathyroid hormone-(1-34) (Potts *et al.*, 1971), and of ACTH-(5-14) (Yajima *et al.*, 1970c). Other ion exchangers have also been used, e.g., Amberlite IRC-50 for bradykinin (Merrifield, 1964b); Amberlite CG-50 for [Phe⁵⁹]-cytochrome c (Sano and Kurihara, 1969), DEAE-cellulose for acyl carrier protein (Hancock *et al.*, 1971; Marshall *et al.*, 1973), and Sephadex C-25SE or Sulfoethyl-cellulose for angiotensin II analogs (Jorgensen *et al.*, 1969, 1970a,b). Specific use of ion-exchange chromatography is made for the removal of truncated sequences carrying acyl terminating groups with additional acidic substituents ("acidic handle"). Wieland *et al.* (1969b) employed Dowex 1 $\times$ 2 to separate 2-carboxy-3-nitrobenzoyl peptides from the desired main product by gradient elution. Amberlite IR-45 served to remove 3-sulfopropionyl peptides effectively (Wissmann and Geiger, 1970).

Gel filtration as the sole means of purification must be judged generally inadequate for many peptides prepared by solid-phase synthesis (e.g., Meienhofer *et al.*, 1970a). It is, however, very useful in combination with other procedures in addition to its role in desalting. For some groups of not too large peptides, specific gel filtration procedures might produce acceptable results. For example, Manning *et al.* (1968b) described the purification of oxytocin and analogs by repeated gel filtration on Sephadex G-15 in (a) 50% acetic acid and (b) 0.2 N acetic acid. Care should be taken to avoid bacterial degradation of synthetic peptides (Vane, 1971) during chromatography. Sephadex gels are ideal media for bacterial growth and should be sterilized at 120° for 25 minutes while suspended in water. Buffers should also be sterilized prior to use (Liu *et al.*, 1971).

Lipophilized (hydroxypropylated) Sephadex LH-20 has been used for several purifications. It seems likely that partition contributes to the efficiency of fractionation in some of the solvent systems that have been used. du Vigneaud and collaborators purified on Sephadex LH-20 deaminooxytocinoic acid dimethylamide (Takashima *et al.*, 1969) and [1-(δ -mercaptovaleric acid)]-oxytocin (Fraefel and du Vigneaud, 1970a) using toluene-glacial acetic acid-water (20:20:3.5), and [1- ω -mercaptoundecanoic acid]-oxytocin using *n*-hexane-toluene-glacial acetic acid-water (50:150:200:35) (Fraefel and du Vigneaud, 1970b). A partially protected linear decapeptide intermediate of gramicidin S (Losse and Neubert, 1970b) was chromatographed on Sephadex LH-20 in dioxane. A homogeneous protected C-terminal eleodoisin heptapeptide amide derivative was obtained from LH-20 in chloroform-acetic acid-methanol (90:5:5) (Halstrøm *et al.*, 1969).

Countercurrent distribution is not only a very powerful fractionation procedure, but it actually allows the state of purity of a peptide to be ascertained by the degree of coincidence of the experimental distribution curve with the theoretical plot.* The procedure has been used for purification of, e.g., bradykinin (Lenard and Robinson, 1967) in 2-butanol-trifluoroacetic acid-water (100:1:100) and for many bradykinin analogs (Stewart and Woolley, 1966), for 5-isoleucine-angiotensin II (Marshall and Merrifield, 1965), for α -ACTH-(4-10) using *n*-butanol-acetic acid-water (4:1:5) (Blake and Li, 1968), for parathyroid hormone-(18-24) using *n*-butanol-acetic acid-water-mercaptoethanol (1200:300:1500:20) (Wang and Merrifield, 1969b), for human hemoglobin β -chain-(124-146) using *sec*-butanol-1% dichloroacetic acid (1:1) (Chillemi and Merrifield, 1969), and for secretin-(1-6) using *n*-butanol-pyridine-acetic acid-water (4:2:1:7) (Ondetti *et al.*, 1968a).

Crystallization does, in our experience with protected nonapeptide intermediates of neurohypophyseal hormones, separate microheterogeneous mixtures more efficiently than many other methods. The power of crystallization of protected nonapeptides from organic solvents has been demonstrated in syntheses of lysine-vasopressin (Meienhofer and Sano, 1968; Meienhofer and Trzeciak, 1971) and of arginine-vasopressin (Meienhofer *et al.*, 1970b, 1971). A protected linear decapeptide hydrazide (gramicidin S intermediate) (Ohno *et al.*, 1971) has recently been prepared. Crystallization from aqueous phase of free peptides after removal of protecting groups has also aided in obtaining homogeneous products. Examples of crystalline peptides (larger

* Theoretical curves may be calculated according to $V = V_{\max} \cdot e^{-\frac{x^2 (K + 1)^2}{2 n K}}$, where $V_{\max}$ = plot of peak tube, x = tube numbers in both directions from peak tube (=0), K = distribution coefficient, and N = numbers of transfer (see Meienhofer, 1967).

than hexapeptides) prepared by solid-phase synthesis include: deamino-oxytocin (Takashima *et al.*, 1968), gramicidin S and an analog (Klostermeyer, 1968; Halstrøm and Klostermeyer, 1968), deamino-oxytocinoic acid methylamide (Takashima *et al.*, 1969), valinomycin (Gisin *et al.*, 1969; Gisin and Merrifield, 1970; Losse and Klengel, 1971), and antamanide and analogs (Wieland *et al.*, 1969a, 1971a).

Other procedures employed for purification include partition chromatography on Sephadex G-25 [*n*-butanol-pyridine-acetic acid-water (30:20:6:25)] (Ondetti *et al.*, 1968a), adsorption chromatography on silica gel (Pereira *et al.*, 1969b; Losse and Klengel, 1971), preparative high-voltage paper electrophoresis (Hörnle, 1967), or preparative paper chromatography (Smith and Dunn, 1970; Smith *et al.*, 1970). Such newly developed powerful fractionation procedures as isoelectric focusing (Finlayson and Chrambach, 1971; Haglund, 1971), rotating free zone electrophoresis (Hjerten, 1970), and helix countercurrent chromatography (Ito and Bowman, 1971) might have considerable potential for solid-phase synthetic products.

Very promising recent developments are affinity chromatography and, so-called, functional purification which have previously been applied mainly to enzymic and immunological work (Cuatrecasas and Anfinsen, 1971a,b). Anfinsen and collaborators and Gutte and Merrifield have used these procedures and achieved highly effective purifications of their synthetic peptide and protein preparations with (partial) staphylococcal nuclease and bovine ribonuclease A sequences, respectively.

3. *Analytic Criteria of Homogeneity*

The requirements for the proof of identity of solid-phase synthetic products with the desired target peptide resemble rather closely those that have been laid down for the characterization of proteins. At least four independent analytic tests should show uniformity (Schultze, 1956; Li *et al.*, 1954; see, also, Meienhofer and Zahn, 1960).

It is widely accepted to date that a correct *amino acid analysis* has, by itself, little significance as criterion for homogeneity of a peptide prepared by the solid-phase method, because (a) contaminating side products might give correct analyses (Meienhofer and Sano, 1968), and (b) the limits of error are too large to detect the presence of less than 5% of failure sequence contamination, even if a missing amino acid residue occurs only once in the entire sequence. However, amino acid analyses must always be obtained and incorrect data would certainly indicate a greatly increased need for purification.

Analytic scale countercurrent distribution, ion-exchange and partition chromatography, gel filtration, and high-voltage electrophoresis have been utilized to assemble criteria for homogeneity of solid-phase synthetic peptides. Of the proven analytic methods, thin-layer chromatography, thin-layer electrophoresis, enzymic digestion (Zuber, 1960), peptide mapping, gas chromatography, and mass spectrometry [and a combination of the latter two (Bayer and Koenig, 1969)] have been applied to examine the products. It appears that some very powerful analytic procedures have not been used thus far to any extent, although they are being routinely used in sequence analysis. End-group analysis [by dinitrophenylation or dansylation (Seiler, 1970) for N-terminals; by hydrazinolysis or carboxypeptidase treatment for C-terminals], and Edman degradation should certainly provide excellent criteria for the homogeneity of synthetic peptides. Solid-phase Edman degradation has been developed (Laursen, 1966; Dowling and Stark, 1969) in which peptides are first attached to a support. The method should be well suited to analyze synthetic peptides prior to removal from the solid support. Gel electrophoresis might also deserve consideration in certain cases. For these methods the reader is referred to Bailey (1967), Hirs (1967), Blackburn (1970), or Needleman (1970).

Frequently quoted as strong argument for molecular identity is complete agreement in biological potency between synthetic and natural materials. Certain reservations toward such claims are indicated since it has become obvious that molecules with considerable sequence variations can exhibit very similar biological activities [striking examples are the species differences in calcitonin structures (Niall *et al.*, 1969)]. Identical biological activities should therefore not be construed, by themselves, as indicating molecular identity (Vane, 1971). On the other hand, this reservation should not be used to argue (without specific, pertinent experimental evidence) that biological activities of synthetic peptides might originate from contaminants which differ from the target sequence. It is possible that some contaminants (failure sequences) might contribute to the biological activity of a microheterogeneous mixture. Such "active" contaminants would each be present in very small percentages, see Fig. 6. It is most unlikely that more than very few failure sequences which are present in a given mixture would possess activity. Therefore, any significant biological potency of the product still has to be attributed largely to the amount of target sequence present in the product mixture. Solid-phase synthesis cannot lead to the discovery of a highly potent failure sequence. To return, however, to the starting discussion, it must be emphasized that biological data should be supplemented by clear and unambiguous chemical and physical criteria for homogeneity before any claims of molecular identity can be convincingly raised.

4. Example

Bovine Pancreatic Ribonuclease A (RNase A). The example, par excellence, for the prospects of protein synthesis by the solid-phase method, is the nuclease preparation by Gutte and Merrifield (1969, 1971) according to the target sequence of bovine pancreatic ribonuclease A. This work illustrates the present scope and limitations of the method. It also shows the correlation of labor and skill invested in actual synthesis on one hand and in purification and characterization of the synthetic product, on the other. [The reading of the article of Gutte and Merrifield (1971) describing their endeavours in purification and characterization of synthetic ribonuclease is imperative for those planning to do solid-phase peptide synthesis.]

After 6 weeks of synthesis, using an electromechanically programmed instrument, enzyme activity was obtained: no doubt, ribonucleases had been made by chemical synthesis. Because of the known microheterogeneous nature of products prepared by the solid-phase method, Gutte and Merrifield (1971) concluded: "We do not suggest that our work, in its present state of development, constitutes a structure proof of ribonuclease A, only that the synthetic molecule bears a close chemical and physical resemblance to the natural protein and that it is a true enzyme with a specific activity not far from that of native ribonuclease A." However, the degree of resemblance can not be established. Therefore, tremendous efforts and much time (several years) were spent to demonstrate that the synthetic product is *not very different* from natural ribonuclease A. In fact, a difference was hardly detectable, except for lower specific activity and for the failure to crystallize. Nevertheless, highly purified synthetic ribonuclease A prepared by the solid-phase method is still a mixture whose contaminating components are so closely related to the main component that they cannot be detected, much less identified, by presently available analytic methods.

Using a programmed schedule (see Section IV,C), 123 cycles gave the protected peptide-resin (Fig. 16). The product was removed from the resin and freed from protecting groups by treatment with anhydrous hydrogen fluoride. Fractionation of the crude mixture on a Sephadex G-75 column by elution with 0.05 *M* ammonium bicarbonate buffer produced three peaks (Fig. 17). Peak II which agreed in its elution pattern with natural RNase A was reduced by mercaptoethanol and reoxidized under optimal conditions for correctly paired disulfide bond formation (White, 1961; Anfinsen and Haber, 1961; Epstein *et al.*, 1962). After desalting by gel filtration on Sephadex G-25 in 0.1 *M* acetic acid, this material, possessing 5% specific activity, was further purified by ion exchange chromatography on Amberlite IRC-50 (Fig. 18). Three peaks were obtained, the first (possessing 13%

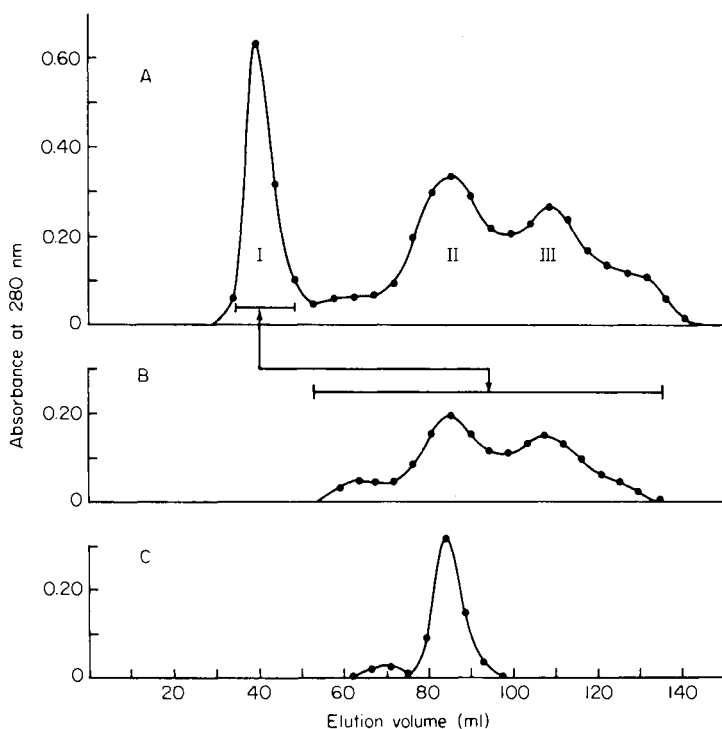


FIG. 17. Gel filtration of the cleaved crude synthetic product and natural RNase A on Sephadex G-75. The column (2.1×45.5 cm) was eluted with 0.05 *M* ammonium bicarbonate buffer. A, 20 mg of crude synthetic RNase A in 1 ml of 0.05 *M* ammonium bicarbonate buffer. Peak I, 5.7 mg; Peak II, 7.5 mg; Peak III, 3.8 mg. B, the synthetic material under Peak I of Chromatogram A was reduced and reoxidized in dilute solution (0.02 mg/ml), and then rechromatographed on the Sephadex G-75 column. C, 5 mg of natural RNase A in 0.25 ml of the eluent. From Gutte and Merrifield (1971).

specific activity) eluted at the same position as reduced-reoxidized natural RNase A. Untreated native natural RNase A eluted at a somewhat different position, but no difference was observed in CM-cellulose chromatography by gradient elution (Fig. 19). Further fractionation was carried out by treatment with trypsin to degrade contaminants with different conformation than that of native RNase A which is known to be very resistant to tryptic digestion (Dubos and Thompson, 1938). Chromatography on Sephadex G-50 gave two peaks. The material from the first peak (exhibiting 61% specific activity) was subjected to ammonium sulfate fractionation (Kunitz, 1940) to produce synthetic product possessing 78% specific ribonuclease

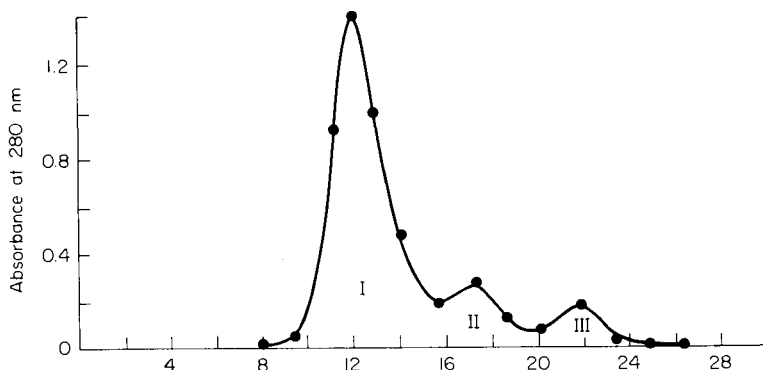


FIG. 18. Ion exchange chromatography of synthetic RNase A on IRC-50. Synthetic material (5 mg) from Peak I, Fig. 17A, was reduced and reoxidized and applied to a 1×25 cm column in 0.25 ml of 0.2 *M* phosphate buffer. The material was eluted with 0.2 *M* phosphate buffer at pH 6.47. Peak I, 3 mg; Peak II, 0.45 mg; Peak III, 0.3 mg. From Gutte and Merrifield (1971).

activity. This is the most active preparation that has thus far been obtained. Analytic evidence for homogeneity was obtained through amino acid analysis, enzyme digestions (complete degradation into amino acids by using first papain and then aminopeptidase M), antibody neutralization, comparative peptide maps, paper electrophoresis, gel filtration, and ion exchange chromatography (as described above). The similarity of the synthetic product to natural RNase A was further shown by close resemblance of substrate specificities and Michaelis constants. Observed differences were the elution positions on Amberlite IRC-50 columns, the somewhat lower specific activity, and the failure, as yet, of the synthetic product to crystallize.

IV. The Operation

The simplicity, the speed, and the ease and convenience of operation are some of the main attractive features of the solid-phase procedure. The manual performance of a cycle for the incorporation of an amino acid residue involves the timed addition of solvents and reagents to a reaction vessel, the timed removal of solutions from the vessel by suction or pressure, and the timed starting and stopping of the stirring mechanism. An untrained person can be instructed to carry out these manipulations by simply following a standard protocol. There is no need for the operator to have experience with solution methods of peptide synthesis to carry out this part of the solid-phase procedure. The manual operation was eliminated and the work load

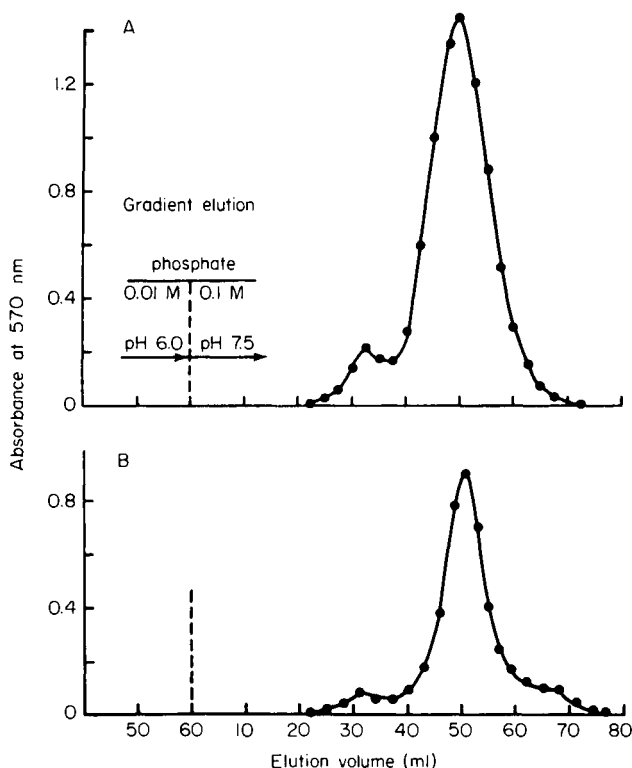


FIG. 19. Ion exchange chromatography of synthetic and untreated natural RNase A on CM-cellulose. After the samples had been applied, the column (1.1×23.5 cm) was eluted with 60 ml of 0.01 *M* phosphate buffer, pH 6.0. Then gradient elution toward 0.1 *M* phosphate buffer, pH 7.5, was started. A, 5.0 mg of untreated natural RNase A; B, 1.9 mg of synthetic RNase A (HBr-HF-treated, fractionated on Sephadex G-75, reduced, and reoxidized), then 0.5-ml (Chromatogram A) and 1.0-ml aliquots (Chromatogram B) of each fraction of the eluate from the column were submitted to alkaline hydrolysis. The hydrolysates were reacted with ninhydrin and the optical densities at 570 nm were measured spectrophotometrically. From Gutte and Merrifield (1971).

further reduced when mechanized instruments were developed which feature control of pumps, timers, and valves through stepping drum or disk programmers. However, these mechanized instruments require an operator possessing experience in both mechanics and electronics.

A number of modified reaction vessels were designed for purposes of improved mixing, temperature control, larger operating capacity, or analytic process control. The requirement for process control became apparent when

standard protocol operations, although adequate for the majority of couplings, resulted sometimes in incomplete reactions. Because of this problem some peptides with particularly unfavorable sequences could not be prepared by the solid-phase method.

Some commercially supplied amino acid derivatives, reagents, and solvents were found to contain impurities which interfered with solid-phase reactions rendering them incomplete. Considerable time and care should therefore be devoted to purification of chemicals. As a result of these developments, even the mere operation of solid-phase synthesis is not necessarily very simple and easy. It requires knowledge in purification of solvents, reagents, and protected amino acids, skill in mechanics and programming, command of analytic control methods, and finally, some experience in handling the products. The speed of operation has also slowed considerably as a consequence of using newly developed analytic process controls. Not infrequently, a cycle requiring several repeat operations and intermediate tests might last days, instead of the 4–5 hours in standard protocolled operations. Thus, a solid-phase synthesis might require considerably more time than expected on the basis of time quotations given in the early literature,* without considering the time required for purification of the product (see Section III,F).

In this section various reaction vessels and stirring devices will first be discussed, followed by a consideration of solvent and reagent quality and procedures for their purification. Several standard schedules for synthesis will be listed which have provided acceptable products. Analytic procedures useful for process control will then be described in some detail. Finally, mechanization and programming of respective machines will be described.

A. REACTION VESSEL AND STIRRING

The problem of designing an efficient vessel and stirring device for solid-phase synthesis resembles that of constructing a good household washing machine! Essential provisions are (a) convenient addition of solvent and reagents, (b) speedy, unobstructed, and as complete as possible removal of solution without any loss of solid, and (c) efficient mixing or circulation of solution through the solid without destructive effects on the solid. Existing designs can be broadly described in four categories; (1) shaking devices, with stirring motion applied from outside the vessel, (2) inside stirring devices, (3) column operation, and (4) centrifugal flow device.

* These favorable time factors are of course real and still apply to the synthesis of the peptides described. Other peptides with favorable sequences can probably be prepared as fast, but again, others will be difficult and slow, and there is no criterion to predict this a priori

1. Reaction Vessels with (Outside) Shaking Devices

The first shaker device and glass vessel for solid-phase peptide synthesis designed by Merrifield (1963) has been described in sufficient detail elsewhere (Merrifield and Corigliano, 1968; Merrifield, 1969a, pp. 266–267; Stewart and Young, 1969, pp. 65–70). This design (Fig. 20) and modified versions are used in most laboratories. The glass vessel is rocked through arcs of 100–110 degrees. Since small amounts of the resin tend to adhere to the upper part of the vessel, above the surface of the liquid, Kusch (1966a) used 360 degree continuous rotation in a similar closed vessel.

Khosla *et al.* (1967b) described a vessel consisting of a roundbottom flask, flattened at the bottom (Fig. 21). Solvents are added, without opening, through a side arm carrying a dropping funnel. Polymer adhering to the walls can be washed down easily. Removal of solutions is effected by suction through a fritted glass disk at the end of a tube reaching to the bottom of the vessel. This vessel can be immersed in ice water with simultaneous shaking for low temperature operations.

Brunfeldt *et al.* (1969a) adapted a 300-ml filter jar with a ground flange

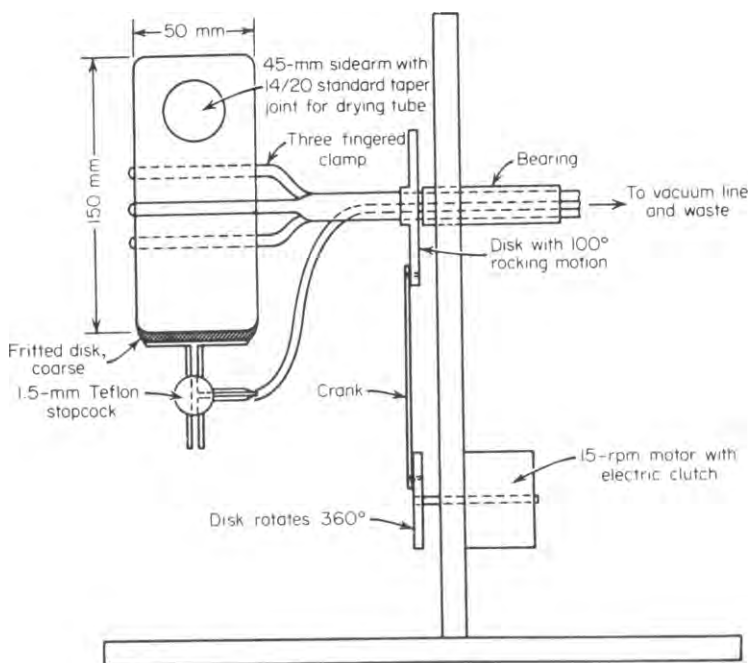


FIG. 20. A manual apparatus for solid-phase peptide synthesis. From Merrifield (1969a).

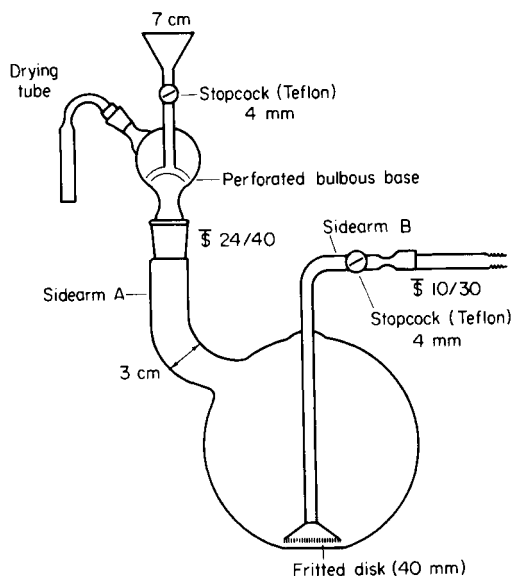


FIG. 21. Reaction vessel utilizing a roundbottom flask for larger amounts of resin. From Khosla *et al.* (1967b).

joint at the top to fit a cover containing two special joints as lead-in for Teflon tubes from a solvent metering flask and a nitrogen pressure valve. Removal of solution through the bottom outlet below the filter disk is effected by nitrogen pressure. The upright reactor is agitated by about 400 strokes per minute with amplitudes variable from 0–50 mm delivered through a shaker arm. (Compare Fig. 22B for a similar device.)

2. Reaction Vessels with Inside Stirring Devices

Internal stirring devices must be designed in such a way so that destructive effects on the swollen polystyrene beads are completely avoided. The model by Brunfeldt *et al.* (1969a) shown in Fig. 22A consists of a 500-ml filter jar with a ground flange joint at the top to fit the cover. The bottom outlet is connected to a circulation pump providing a continuous washing of the reactor wall during the reactions. The cover contains three inlets for Teflon tubing connected to the solvent metering flask, the circulation pump, and a nitrogen pressure valve, respectively. In the center of the cover a ground joint accepts a Quick-fit stirring head which guides the propeller stirrer with which the agitation of the reaction mixture is performed. Above the highest liquid level in the reactor a Teflon disk is attached to the stirrer to eject the circulating liquid onto the reactor wall. A similar (full-flow) re-

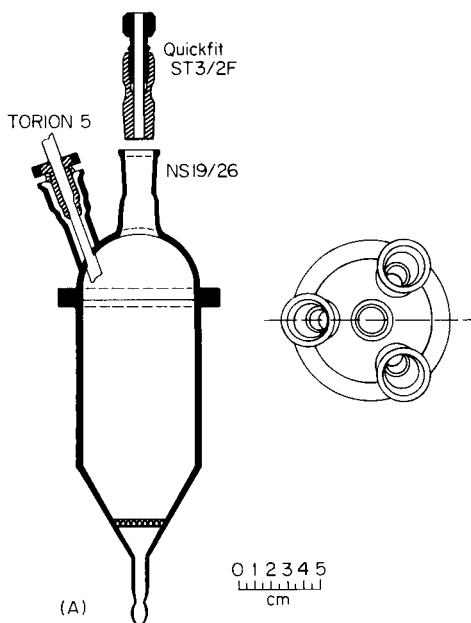


FIG. 22. Reaction vessels for solid-phase peptide synthesis. (A) Vessel providing agitation by internal stirring. (In the sectional drawing only one of the three inlets is shown.) From Brunfeldt *et al.* (1969a). (B) Vessel with inside stirring. From Grahl-Nielsen and Tritsch (1969). (C) Conical vessel. From Mizoguchi *et al.* (1970).

action vessel, but jacketed for thermostatically controlled kinetic studies, has been designed by Beyerman and Hirt (Beyerman, 1972).

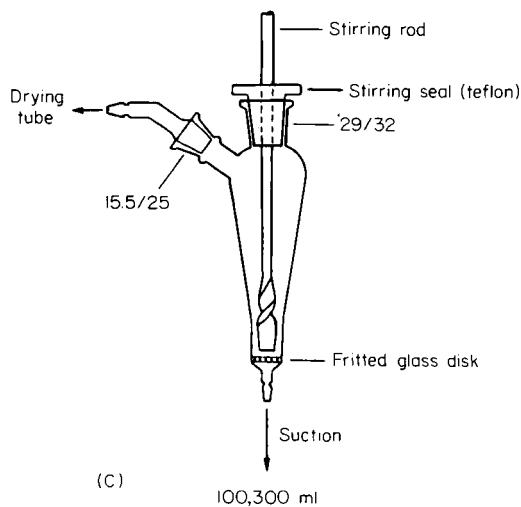
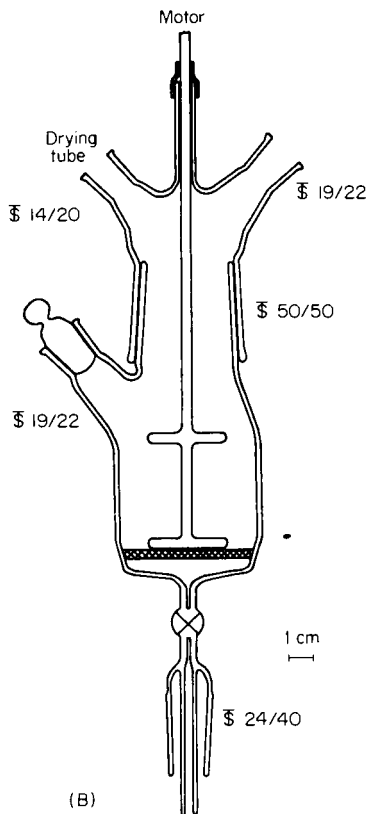
Another vessel, with stirrer, has been described by Grahl-Nielsen and Tritsch (1969). It consists of a glass cylinder (250-ml volume) with a side arm and a fritted glass disk near the bottom (Fig. 22B). The outlet below is fitted to a vacuum reservoir. An inner glass joint at the upper end of the vessel holds the lid which has three openings, for the stirrer, for a drying tube, and for solvent and reagent addition, respectively.

A still simpler design with a mechanical stirrer is shown in Fig. 22C (Mizoguchi *et al.*, 1970). Solvents and reagents are added manually through the side arm which can be opened by removing the drying tube.

A different type of internal stirring, percolation by a gentle upward flow of nitrogen through the fritted plate near the bottom of a conical reaction vessel (Gut and Rudinger, 1968) or of cylindrical vessels (Loffet and Close, 1968; Dorman and Markley, 1971), has also been used.

3. Column Operation

Bayer *et al.* (1970c) pointed out that an automated synthesis of polypeptides by the solid-phase method would be much easier to operate if



the support was in column form. With the resin commonly used, this is not possible because the different solvents used in a synthetic cycle cause variable swelling of the resin (see Table VII). Therefore, inorganic supports such as porous glass, silica gel, or quartz have been investigated and so-called brush-type solid supports have been developed (Bayer *et al.*, 1970c; Parr and Grohmann, 1971). The synthesis of several peptides by column operation in a specially designed synthesizer showed that reaction times could be reduced by a factor of two or more. It will be interesting to see further developments in this area.

4. Centrifugal Flow Device

An apparatus which combines the advantages of the column procedure (i.e., continuous flow) with those of the batch procedure (i.e., efficient removal of liquid) has recently been designed (Birr and Lochinger, 1971; Birr *et al.*, 1973). The resin is contained in a rapidly rotating porous Teflon beaker which is mounted inside a slightly larger closed glass housing (Fig. 23). Reagent solutions or washing media are introduced into the vessel (Fig. 23A) by tubes fitted through a screw cap top. Centrifugal force moves the resin so as to be located along the beaker walls and drives the liquid through the resin and the porous wall of the spinning rotor to the inner wall of the glass housing which it climbs and from there plunges back into the rotating centrifuge basket (Fig. 23B). A very swiftly rotating current penetrates the resin, and none of the resin particles can avoid contact with the liquid. There is no abrasive action on the resin by the porous Teflon filter and no danger of blockage. When the outlet of the reactor is opened, the rotor empties very quickly and completely by centrifugal force (Fig. 23C). A

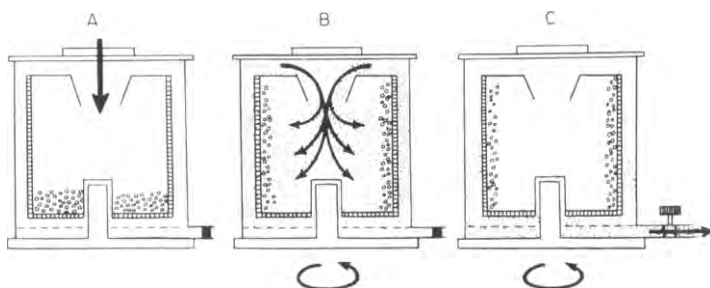


FIG. 23. Centrifugal flow reactor. Rotating internal beaker possesses walls of porous teflon. (A) Filling stage. Reagents and solutions enter the vessel through teflon tubes at the top. (B) Rotating and mixing stage. The resin is located at the porous walls and a stream of liquid circulates intensively reaching all resin particles. (C) Emptying stage. The bottom outlet is opened and the liquid is emptied spontaneously by centrifugal force. From Birr and Lochinger (1971).

complete cycle for the incorporation of one amino acid residue into the growing peptide chain requires approximately 165 minutes. This vessel and its operation appear to be very efficient.

B. SOLVENTS AND REAGENTS

Although Merrifield recommends the use of commercial analytic grade solvents and reagents (Merrifield and Corigliano, 1968; Felix and Merrifield, 1970) many other workers believe that only highly purified reagents and carefully distilled anhydrous solvents must be used to obtain acceptable results (Mizoguchi and Woolley, 1967; Klostermeyer, 1968; Wieland *et al.*, 1969a, 1971c; Krumdieck and Baugh, 1969; Meienhofer *et al.*, 1970b; Polzhofer and Ney, 1970a; Loffet *et al.*, 1971). It has been strongly emphasized that the degree of dryness and purity of solvents and reagents is of decisive importance (Wieland *et al.*, 1969a, 1971c; Polzhofer and Ney, 1970a). The necessity of using highly purified Boc-amino acids was first pointed out by Bayer *et al.* (1968a) who scrutinized their preparations for homogeneity by mass spectrometry, gas chromatography, polarimetry, and thin-layer chromatography. Physical data for purified Boc-amino acids have been listed by Bayer *et al.* (1968a), by Beyerman *et al.* (1968a), and by Halstrøm *et al.* (1969).

Very recently attention has been drawn to the limited stability of some of the most frequently used solvents. Methylene chloride releases hydrogen chloride slowly but steadily; chloroform also releases hydrogen chloride at a somewhat faster rate (Wieland *et al.*, 1971c). Triethylamine in methylene chloride seems to be particularly unstable and triethylammonium chloride deposits have been observed.* These solvents or solutions should, therefore, always be prepared freshly. Passage of distilled methylene chloride or chloroform through columns of basic aluminum oxide to remove hydrogen chloride and storage in brown bottles has been recommended (Wieland *et al.*, 1971c). We have found that even analytic or reagent grade solvents as well as dicyclohexylcarbodiimide are not pure enough for use without prior purification—in fact, occasional lots (by the same supplier) were highly contaminated. Purification procedures routinely used in our laboratory are outlined below.

Ammonia: From gas cylinder (Matheson Gas Co., East Rutherford, New Jersey). Passed through a drying flask filled with KOH pellets, and distilled from sodium, if necessary.

Dicyclohexylcarbodiimide: Distilled *in vacuo* under nitrogen and stored at 4°.

* Discussions at the *Eleventh European Peptide Symposium*, Vienna, 1971.

Hydrogen bromide: From gas cylinder (Matheson). Bubbled through a solution of resorcinol in trifluoroacetic acid. Cylinder storage should not exceed 6 months (Matheson Catalog).

Hydrogen fluoride: From gas cylinder (Matheson). Dried over CoF_3 .

Triethylamine: Distilled from ninhydrin (1 gm/liter) to remove primary and secondary amines. Redistilled from sodium wire under nitrogen and stored in brown bottles under nitrogen.

Trifluoroacetic acid: Distilled from P_2O_5 . Redistilled from dried valine to remove traces of anhydride.

For the preparation of hydrogen chloride in glacial acetic acid and of 4 *N* hydrogen chloride in dioxane, see the book by Stewart and Young (1969, pp. 30–31) where experimental details have been described.

Acetic acid (glacial): Acetic anhydride (1 ml/liter) is added to react with the water present. Heated for 1 hour at about 110° (just below boiling) in the presence of 20 gm CrO_3 /liter. Distilled at constant boiling point.

Chloroform: Distilled in the dark from anhydrous CaSO_4 or from P_2O_5 . Passed through a column of basic aluminum oxide (Woelm, No. W200, Waters Associates Inc., Framingham, Massachusetts 01701). Stored in brown bottles over Linde 4A molecular sieve.

Dimethylformamide: Distilled from ninhydrin (1 gm/liter) *in vacuo* (1–2 mm Hg) under nitrogen. Stored in brown bottles over Linde 4A molecular sieve. The solvent can also be purified by treatment with CaO (A. B. Thomas and Rochow, 1957).

Dioxane: Distilled from LiAlH_4 under nitrogen in the dark. Stored in brown bottles under nitrogen. Can be freed from peroxide by passage through a column of aluminum oxide (Merck No. 71707).

Methylene chloride: Same treatment as chloroform.

C. SCHEDULE FOR SYNTHESIS

The repetitive nature of operations in solid-phase synthesis lends itself to the formulation of "standard" or "general" procedures, and many reports refer to the use of one which was developed by Marshall and Merrifield (1965). Variations were made as required for different coupling methods and uses of different α -amino protecting groups. When a programmed mechanized instrument was developed in pursuit of the original intention (Merrifield, 1966) of the solid-phase concept, essentially two standard procedures were recommended (Merrifield *et al.*, 1966), one for the use of Boc-amino acids and dicyclohexylcarbodiimide, and the other for Boc-amino acid *p*-nitrophenyl esters. As experience accumulated over numerous solid-phase syntheses and, especially, as analytic controls were developed, a

Table XVI—The “Standard” Marshall-Merrifield (1965) Schedule (Angiotensin and Bradykinin Peptides)

Operation no	Reagent, solvent ^a	Time (minutes)
1	Glacial acetic acid wash (3 times) ^b	30
2	1 <i>N</i> HCl in glacial acetic acid ^c	
3	Glacial acetic acid wash (3 times)	
4	Ethanol wash (3 times)	
5	Dimethylformamide wash (3 times)	10
6	Triethylamine in dimethylformamide (1:10)	
7	Dimethylformamide wash (3 times)	
8	Methylene chloride wash (3 times)	
9	Boc-amino acid (4 equiv) in methylene chloride	10
10	Dicyclohexylcarbodiimide (4 equiv) in methylene chloride	120
11	Methylene chloride wash (3 times)	120
12	Ethanol wash (3 times)	

^a For 1 gm of resin 6 ml of solvent was used.

^b A wash operation involves 1–3 minutes shaking.

^c 4 *N* HCl in dioxane has later been recommended (Merrifield, 1967).

growing number of reports appeared describing erratic (sequence-dependent) failures or part failures of standard procedures in individual coupling steps. In the wake of these observations of ubiquitous and unpredictable hazards, confidence in a single or a few general procedures has considerably weakened, and consequently many modifications or alternatives have been suggested. At present one cannot recommend any standard procedure for exploratory (first) syntheses of novel peptides. For such project an appropriate combination of analytic controls should be applied to each individual step. This will enable the investigator to recognize which cycles of his synthesis proceed well and which give rise to difficulties demanding special treatment. Based on such step by step evaluation, a sort of “species-specific” procedure may be formulated which could be useful for a program of analog preparation.

The following description of a number of prototype procedures should be viewed, in this context, as having provided acceptable results in syntheses of particular groups of peptides. Only the repetitive cycles for the incorporation of amino acid residues are described here. The initial and final stages of the solid-phase method, attachment of the first amino acid to the resin, and removal of the solid support from the final product are not as routinely or schematically applicable as the cycles for the peptide chain elongation. The protocol shown in Table XVI has been employed by Marshall and Merri-

Table XVII—Schedule for the Use of Active Esters ^a

Operation no.	Reagent, solvent ^b	Time
1	Glacial acetic acid wash (3 times)	30 minutes
2	1 <i>N</i> HCl in glacial acetic acid	
3	Glacial acetic acid wash (3 times)	
4	Ethanol wash (3 times)	
5	Dimethylformamide wash (3 times)	10 minutes
6	Triethylamine in dimethylformamide (1:5)	
7	Dimethylformamide wash (5 times)	
8	Boc-amino acid <i>p</i> -nitrophenyl ester (equiv) in dimethylformamide ^c	12–16 hours
9	Dimethylformamide wash (3 times)	
10	Ethanol wash (3 times)	

^a Employed by Hörnle (1967) for a solid-phase synthesis of bovine insulin A-chain with the exclusive use of *tert*-butoxycarbonyl *p*-nitrophenyl esters.

^b For 1 gm of resin 8 ml of solvent were used, except in operation 8.

^c Solvent volume was 4 ml/gm resin.

field (1965) in syntheses of angiotensins. Various solvents are used for washing which cause the resin to swell (dimethylformamide, methylene chloride) and shrink (ethanol), successively. A 2-hour coupling period is used. This schedule has since been used in numerous other syntheses and is commonly referred to as the “standard procedure.”

The solubility properties of Boc-amino acid *p*-nitrophenyl esters do not allow the general use of methylene chloride as a solvating medium in the coupling step. Dimethylformamide is commonly used in active ester condensations. It swells the polystyrene resin almost as much as methylene chloride (see Table VII). Table XVII depicts the schedule used by Hörnle (1967) for a synthesis of bovine insulin A-chain with the exclusive use of *tert*-butoxycarbonylamino acid *p*-nitrophenyl esters.

The procedure of Manning *et al.* (1970) and Baxter *et al.* (1969b), outlined in Table XVIII, was applied to syntheses of a number of oxytocin analogs, among them the extraordinarily active [4-threonine]-oxytocin. Manning has commented (Manning and Sawyer, 1972) that this method, in their hands, is by far the best for making peptides of this type.*

A seemingly very useful additional operation, especially in view of the instability of methylene chloride and chloroform [which both continuously liberate small amounts of hydrogen chloride (Wieland *et al.*, 1971c)], has

* Dr. Manning has provided us with samples of some protected nonapeptide intermediates. The very high degree of purity of these samples is, indeed, impressive.

Table XVIII—Protocol of Manning and Collaborators (1970) in which All Major Steps of Deprotection, Neutralization, and Coupling are Carried out Twice

Operation no.	Reagent, solvent ^a	Time (minutes)
1	Methylene chloride wash (3 times)	15 (total)
2	Methylene chloride-ethanol (1:1) wash (once)	
3	Ethanol wash (3 times)	15
4	Ethanol-glacial acetic acid (1:1) wash (once)	
5	Glacial acetic acid wash (3 times)	15
6	1 <i>N</i> HCl in glacial acetic acid ^b	5
7	1 <i>N</i> HCl in glacial acetic acid ^b	25
8	Glacial acetic acid wash (3 times)	15
9	Glacial acetic acid-ethanol (1:1) wash (once)	
10	Ethanol wash (3 times)	
11	Ethanol-chloroform (1:1) wash (once)	
12	Chloroform wash (3 times)	15
13	Triethylamine in chloroform (1:15)	5
14	Chloroform wash (once)	
15	Triethylamine in chloroform (1:15)	5
16	Chloroform wash (3 times)	15
17	Chloroform-methylene chloride (1:1) wash (once)	
18	Methylene chloride wash (3 times)	15
19	Boc-Amino acid (3 equiv) in methylene chloride ^c	20
20	Dicyclohexylcarbodiimide (3 equiv) in methylene chloride ^c	4 hours
21	Methylene chloride wash (3 times)	15
22	Methylene chloride-chloroform (1:1) wash (once)	
23	Chloroform wash (3 times)	15
24	Chloroform-methylene chloride (1:1) wash (once)	
25	Methylene chloride wash (3 times)	15
26	Boc-Amino acid (3 equiv) in methylene chloride	20
27	Dicyclohexylcarbodiimide (3 equiv) in methylene chloride	8 hours

^a For 1 gm of resin 10 ml of solvent were used.

^b For the deprotection of Boc-glutamyl residues anhydrous trifluoroacetic acid was used (Manning, 1968; Takashima *et al.*, 1968).

^c Boc-Asparagine and Boc-glutamine were incorporated by the *p*-nitrophenyl ester coupling. This led to elimination of steps 20–27 and the replacement of methylene chloride by dimethylformamide in steps 17–22 and 24–26.

been described by Markley and Dorman (1970). They treated the resin after the coupling reaction with triethylamine in methylene chloride to reconvert into free amine any residual unreacted amino groups that could have become protonated after the original neutralization. Residual amine is thus freed for a repeated coupling (or for a terminating acylation, see Section III,D,1). Occasionally ethanol wash operations have been carried out before or after the triethylamine treatment. This should be avoided since traces of alcohol and amine could cause base catalyzed alcoholysis of the peptide to resin linkage resulting in loss of peptide.

The schedule of Gutte and Merrifield (1969, 1971) (Table XIX) has been designed for use with a stepping drum programmer. In this schedule the resin is kept in a highly swollen state in methylene chloride or chloroform throughout the deprotection and coupling operations. Only once, at the end of the cycle, ethanol washing is used which shrinks the resin. A reaction time of 5 hours is allowed for carbodiimide mediated peptide bond formation.

As pointed out above, the schedules for solid-phase operations given in Tables XVI to XIX should be regarded as useful for the synthesis of

Table XIX—Programmed Procedure of Gutte and Merrifield (1969, 1971)^a

Operation no.	Reagent, solvent ^b	Time (minutes)
1	Methylene chloride wash (twice)	2 (each)
2	Trifluoroacetic acid in methylene chloride (1:1)	2
3	Trifluoroacetic acid in methylene chloride (1:1)	21
4	Methylene chloride wash (5 times)	2 (each)
5	Chloroform wash (3 times)	2 (each)
6	Triethylamine in chloroform (1:10)	7
7	Chloroform wash (3 times)	2 (each)
8	Methylene chloride wash (3 times)	2 (each)
9	Boc-Amino acid (3 equiv) in methylene chloride ^c	7
10	Dicyclohexylcarbodiimide ^d (3 equiv) in methylene chloride	300
11	Methylene chloride wash (twice)	2 (each)
12	Ethanol wash (3 times)	2 (each)

^a Used for preparing enzymes with target sequence of bovine ribonuclease A.

^b For 1 gm of resin 12.5 ml of solvent were used.

^c Because of their low solubility in methylene chloride, Boc-Arg(NO₂) and Boc-His (5 equiv used) were first dissolved in dimethylformamide (4 vol) and then diluted with methylene chloride (1 vol) before adding to the peptide-resin.

^d Boc-asparagine and Boc-glutamine were coupled as *p*-nitrophenyl esters (4 equiv) in dimethylformamide, employing 10 hour reaction periods.

those particular peptides (or classes of peptides) that have been prepared by following these protocols. Different peptides might require different or modified procedures which should be elaborated individually.

An open question in this context is which quantities of resin might be handled successfully in a single run. Up to 30 gm resin have been used by Ney and Polzhofer (1968) in a flask-type vessel (Fig. 22A) for the preparation of casein-related peptides, and also by Fankhauser *et al.* (1973) for a synthesis of hexaleucine. The upper limits have probably not yet been reached.

D. ANALYTIC CONTROL

Although the solid-phase method depends basically on quantitative or near quantitative reactions, it has been shown above that this requirement is frequently not attained. The timely recognition of incomplete reactions has, however, for several years been impeded by the lack of effective means of analytic control during the course of a synthesis. This meant that the investigator knew nothing about the progress of his synthesis until the very end. Consequently, considerable efforts have been made to develop sensitive controls for solid-phase synthesis. It was soon recognized that amino acid analysis, the most powerful analytic tool in solution synthesis, is of limited value for three reasons: (a) it has to be done after each cycle for meaningful interpretation, (b) errors due to hydrolysis (incompleteness, decomposition) are greatly enhanced with resin-peptides,* and (c) the time required (2–3 days) is incompatible with one of the premises of the method: speed. Merrifield (1967) has, however, used amino acid analysis after each cycle to examine the reasons for an observed abrupt partial chain termination during a synthesis of angiotensinylbradykinin (Section III,D,1, Fig. 12). Another type of control described by Merrifield (1963) involves determination of halogen by Volhard titration after the deamination step.

During the past few years a number of relatively more effective analytic controls have been developed (for a recent discussion, see Beyerman *et al.*, 1973a). For the most part, these are titrimetric or colorimetric procedures but radioactively labeled protecting groups or Edman degradation combined with mass spectrometry have also been described. Most of these methods involve the determination of "total amine" content after cleavage of the α -amino protecting groups or of "residual amine" content, due to incomplete peptide bond formation. Several procedures permit the continuous monitor-

* Polzhofer and Ney (1970b), for example, observed the formation of glutamic acid γ -ethyl ester and of γ -(2-hydroxyethyl) ester during hydrolysis of resin-bound (or free) glutamic acid γ -benzyl ester in HCl–dioxane.

ing of the progress of coupling reactions through labeled or chromophoric reaction components. A shortcoming of several of these analytic control procedures is that they are not done on the peptide-resin itself but rather on products released into the embedding solution. Such indirect determinations are inherently less accurate.

1. Titrimetric Methods

The first systematic determination of total amine content after deprotection in each cycle was carried out by Kusch (1966a), who found that direct titration of peptide resin samples in phenol-ethanol (8:2) with HCl permitted the quantitative determination of free amino groups. Potentiometric titration of the entire peptide-resin inside the reaction vessel as part of the synthetic schedule has been described by Brunfeldt *et al.* (1969b, 1971). Following deprotection, titration of total amine with perchloric acid (0.1 *N*) in methylene chloride-glacial acetic acid (1:1) required 1 hour. The necessary triethylamine treatment and washings to remove the perchloric acid lasted 0.5 hour. Titration of residual amine after coupling took 0.5 hour. Consequently, one synthesis cycle was extended by about 2 hours, which is acceptable in view of the importance of obtaining an analytic process control.

Volhard chloride titration to determine the amount of triethylammonium chloride obtained from the neutralization reaction after deprotection has been described by several investigators (Merrifield, 1963; Bayer and Hagenmaier, 1968; Bayer *et al.*, 1968a; Okuda and Zahn, 1969) as a measure of the total amine content of the peptide-resin. This control has been employed and recorded through an entire solid-phase synthesis of apoferreredoxin involving 54 synthetic cycles (Bayer *et al.*, 1968a), see Fig. 10. Visser *et al.* (1968) used the Mohr chloride determination for the same purpose. Obviously, these controls are not applicable when trifluoroacetic acid is used for deprotection. In these instances the procedure of Dorman (1969) can be used, which was developed for the determination of residual amine after coupling. The entire peptide-resin inside the reaction vessel is treated with 0.3 *M* pyridinium chloride in methylene chloride forming the hydrochloride of uncoupled resin amine. Neutralization and washing as usual is followed by potentiometric chloride titration using silver nitrate.*

2. Colorimetric Methods

Kaiser *et al.* (1970) described a rapid ninhydrin test for qualitative estimation of the completeness of amino acid coupling at each step. A few

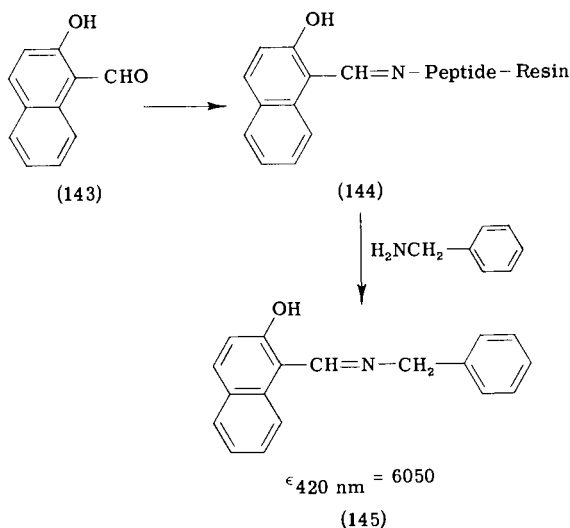
* The Dorman-titration cannot be used for peptide-resins containing histidine residues (Schaich and Schneider, 1973). Both free and *N*¹-benzyl protected histidine residues can be protonated by pyridinium hydrochloride.

Table XX—Estimation of Residual Amine on Peptide-Resin by Ninhydrin Color ^a

% Residual amine ^b	Ninhydrin color test ^c
24	Beads dark blue—dark blue solution
16	Beads dark blue—moderately blue solution
6	Beads moderately blue—light blue solution
0.6	Beads light blue—light greenish solution
0	Beads white—yellow solution

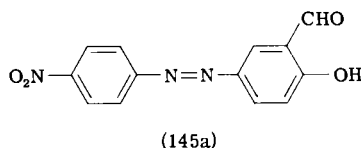
^a After Kaiser *et al.*, 1970.^b Based on a total peptide content of 0.52 mmole/gm resin.^c With N-terminal prolyl or β -benzyl aspartyl residues brown to reddish brown colors are observed.

drops of ninhydrin reagent (in phenol-ethanol, containing a trace of KCN) are added to a small sample of resin (5–10 mg) in a test tube which is heated for 5 minutes at 100°. Color intensities and shades of the resin beads and of the solution indicate certain quantities of free amine (see Table XX). The great advantage of this test is its rapidity. It requires about 15 minutes and is thus the fastest of all presently known control procedures for solid-phase synthesis. It is also very reliable with a sensitivity of about $\pm 1\%$. The procedure has recently been quantitated by Chou *et al.* (1971) to determine total amine after deprotection. Staining of peptide-resin beads with bromocresol purple has been used by Beyerman and Hindriks (1971) to visually estimate the presence or absence of residual free amine. Similarly 15-dimethylaminonaphthalene-1-sulfonyl chloride (dansyl chloride) or 2,4-dinitrofluorobenzene have been used (Zhukova *et al.*, 1970).



Esko *et al.* (1968) determined residual free amino groups by converting them into Schiff bases (144) through reaction with 2-hydroxy-1-naphthaldehyde (143). By treatment of the azomethine (144) with an excess of benzylamine, N-(2-hydroxy-1-naphthylidene)-benzylamine (145) is released into the solution and determined by its extinction at 420 nm. The sensitivity of this procedure was determined to be about $\pm 3-4\%$ (Esko and Karlsson, 1970). It has been used for kinetic studies, e.g., of the coupling of Boc-asparagine *p*-nitrophenyl ester to peptide-resin (Karlsson *et al.*, 1970a). Li and Yamashiro (1970) used the method to determine coupling yields in 80 of the 187 cycles during the preparation of a protein with human growth hormone (original sequence) as the target sequence.

The method of Esko *et al.* (1968) has recently been modified by Losse and Klengel (1971) to provide a qualitative visual test. Reaction of *p*-nitrophenylazo salicylaldehyde (145a) with residual amine on peptide-resin produced a deep red color.



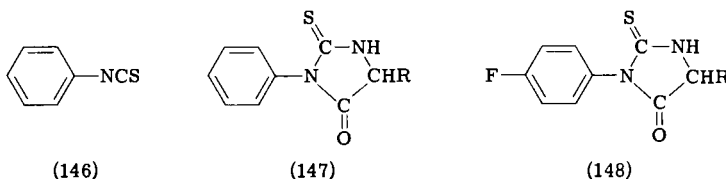
N α -(2-Hydroxyl-1-naphthal)amino acids (100) (Sheehan and Grenda, 1962) have also recently been applied to peptide chain elongation in solution and solid-phase synthesis (Khosla *et al.*, 1972). For the latter the pentachlorophenyl esters were used and the progress of the reaction during the coupling step was monitored by measurement of the decrease in optical density of the solution at 420 nm. Deprotection, using benzylamine, was also quantitatively analyzed as described by Esko *et al.* (1968).

Activating or protecting substituents which possess chromophoric character or give rise to chromophoric leaving groups during coupling or deprotection lend themselves ideally to use in control procedures. The photometric measurements of 5-chloro-8-hydroxyquinoline at 330 nm (Rudinger and Gut, 1967) or of *p*-nitrophenol at 314 nm (Bodanszky and Sheehan, 1966; Hörnle, 1967; Weitzel *et al.*, 1968a; Jakubke and Baumert, 1973) have been used. Biphenyl dimethyl carbinol and 2-(*p*-diphenyl)propene are formed during acidolytic cleavage of the 2-(*p*-biphenyl)isopropoxy-carbonyl protecting group (Sieber and Iselin, 1968b). Their extinction at 254 nm has been measured to determine the progress and completeness of deprotection of Bpoc-peptide-resins. This control has recently been used by Yamashiro *et al.* (1972) and by Wang and Merrifield

(1971).^{*} Photometry at 389 nm has served for kinetic studies of the coupling of *o*-nitrophenylsulfenylamino acids to peptide-resin, for the control of the subsequent removal of the N-protecting group (Gut and Rudinger, 1968), and for kinetic studies on the removal of Nps-peptides from the solid-support by alcoholysis (Beyerman, 1972).

3. Other Methods

A different approach for the determination of residual amine has been developed by Weygand and Obermeier (1968). Edman degradation was combined with a mass spectrometric dilution analysis of phenylthiohydantoins. A dried sample of peptide resin is treated with a large excess of phenylisothiocyanate (146) and then subjected to the general Edman procedure (Blömbäck *et al.*, 1966). To the resulting solution containing the phenylthiohydantoin (147) of the N-terminal amino acid of the amine component a defined amount of the corresponding *p*-fluoro substituted phenylthiohydantoin (148) is added for mass spectrometry. The ratio of the two mass peaks determines the amount of residual unreacted amine in the peptide resin.



Geising and Hörnle (1972) have combined Edman degradation with scintillation counting of the [¹⁴C]-labeled thiazolinone or phenylthiohydantoin derivatives to develop a sensitive resin-amine analysis which was applied to determinations of total amine and residual amine contents of resin samples before and after the coupling step (Hörnle and Geising, 1971). Radioactively labeled α -amino protecting groups appear to be ideally suited for process control in solid-phase synthesis. The coupling step could be monitored by the decrease of activity in the reaction solution and the deprotection step by the activity released from the peptide-resin into solution and by the disappearance of activity from the solid. Such a technique has first been described by Krumdieck and Baugh (1969), who employed Boc-glutamic acid α -benzyl ester, which was labeled with ¹⁴C in position 2 of

^{*} Southard *et al.* (1971c) have, similarly, followed the cleavage of the 2-benzoyl-1-methylvinyl protecting group (Southard *et al.*, 1969, 1971a) by spectrophotometry of the resulting benzoyl acetone.

the *tert*-butoxycarbonyl group, to monitor syntheses of pteroylloligo- γ -glutamates.

Recently, Beyerman *et al.* (1973a) have extended this procedure to include many other amino acids, and they plan to develop the method into a continuous process control for fully automated solid-phase synthesis. Total amine was also determined (Beyerman *et al.*, 1973a) by treating small resin samples with a twofold excess of [^{35}S]-sulfuric acid (0.1 *M* in glacial acetic acid, 3 μCi) and measuring the decrease of radioactivity in solution.

Radioactively labeled terminating agents, [^{14}C]-*N*-acetylimidazole or [^{14}C]-acetic anhydride, have been adopted for amine determination, both before and after the coupling step (Fankhauser *et al.*, 1973). After treatment with the labeled reagents, weighed samples of dried peptide-resin were combusted and the [^{14}C]- CO_2 counted in a liquid scintillation counter to provide a measure of resin amine content.

Bayer and collaborators (1971) have proposed the use of fluorine nuclear magnetic resonance spectroscopy to scrutinize the homogeneity of solid-phase synthetic peptides. For this purpose the peptides have to be trifluoroacetylated and esterified. So far the method has only been used to identify individual components in mixtures of trifluoroacetyl-dipeptide esters (Bayer *et al.*, 1971b).

It is evident that the numerous efforts to develop sensitive and reproducible analytic procedures provided several useful techniques. However, they all suffer from inadequate sensitivity. As discussed in Section II,C, solid-phase peptide synthesis should aim at achieving eventually 99.9% yields or more both in coupling and deprotection. Analytic procedures must therefore possess at least 0.1% or better sensitivity. Fankhauser *et al.* (1973) have calculated standard deviations for a few widely used methods, Table XXI. The reproducibility lies generally between 1 and 10% which means that none of these procedures is sensitive enough. Misleading results have been obtained which seemed to indicate a failure-free ideal progress of a synthesis. In reality, reactions were quite incomplete. This became evident when several different procedures were used for product analysis in a comparative manner.

Nevertheless, control of all reactions throughout a solid-phase synthesis is strongly recommended whenever new peptides are synthesized. A judicious combination of several different analytic procedures (e.g., Mehlis *et al.*, 1971) should considerably increase the overall accuracy. The rapid ninhydrin color test of Kaiser *et al.* (1970), which discriminates between complete or incomplete coupling, might, for instance, be used in combination with one of the titrimetric or colorimetric determinations of total amine content to assure complete deprotection. Additional valuable information con-

Table XXI—Reproducibility of Analytic Methods for the Determination of Amine Contents in Peptide-Resins ^a

Method	Sample size	Range of resin substitution (μ mole/gm)	Estimated standard deviation (%)
Potentiometric chloride titration (TEA $\times$ HCl)	30 gm	400–900	0.5
	0.1 gm	400–900	1.5
Pyridine chloride incubation—titration	1 gm	50–100	5–10
[¹⁴ C]-Acetylimidazole	2–10 mg	400–900	1.5
[¹⁴ C]-Acetic anhydride	10–20 mg	10–30	7
2-Hydroxy-1-naphthaldehyde	} 2–10 mg	400–900	1–3
—Benzylamine		0.5–2	10–20

^a From Fankhauser *et al.*, 1973.

cerning the progress of a synthesis can be obtained by cleaving peptide samples from the solid support and examining chromatographic or electrophoretic properties. In this context a suggestion by Karlsson *et al.* (1970b) has so far not received the attention which it appears to merit. These authors isolated the small amounts of peptide which were lost from the resin during each deprotection step, using trifluoroacetic acid in methylene chloride (1:1), and utilized the material for analytic purposes. Considerable amounts of valuable synthetic material could thus be saved. For example, as much as 48% of the overall yield was sacrificed for analytic purposes by Li and Yamashiro (1970) in a synthesis which proceeded through 187 cycles.

E. MECHANIZATION AND PROGRAMMING

The automation of peptide synthesis was the original incentive for Merrifield in his endeavours to develop the solid-phase method. The design and construction of a mechanized programmed machine and operating instructions are described in the following literature: Merrifield (1965a, 1966, 1967, 1968b, 1969b), Merrifield and Stewart (1965), Merrifield *et al.* (1966), and Stewart and Young (1969). The apparatus developed by Merrifield, Stewart, and Jernberg (1966) features two main components, a control system and a system for the chemical reactions. A stepping drum programmer controls the operations of the shaker for the vessel, a solution pump, distributor valves, air vent, vacuum valves, and timers. The other main component comprises the reaction vessel, the distributor valves for solvents and reagent solutions, and the storage bottles for these liquids as shown schematically in Fig. 24. The operation, as described in detail by

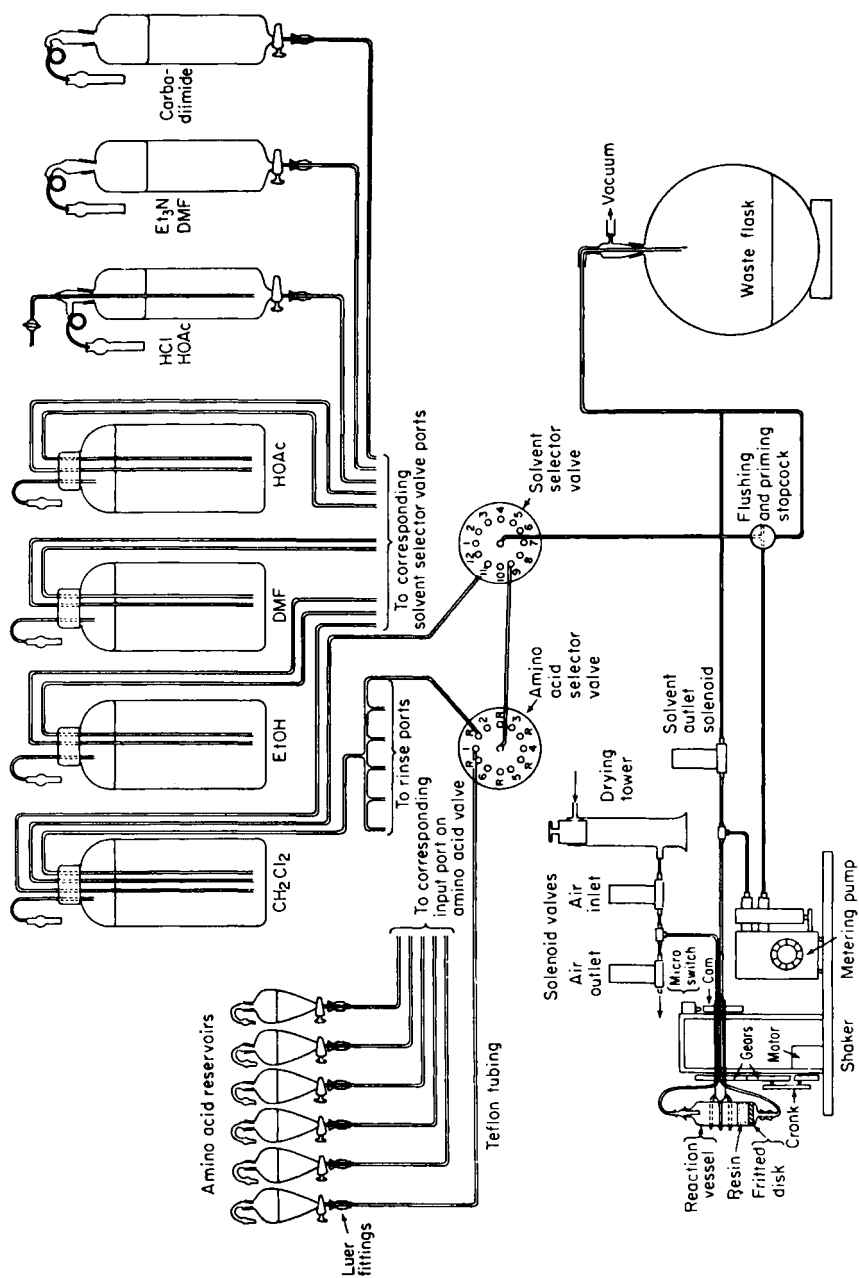


Fig. 24. Schematic drawing of an apparatus for "automated" synthesis of peptides. From Merrifield *et al.* (1966).

Merrifield *et al.* (1966) is essentially a mechanized sequence of actions proceeding strictly according to a predetermined schedule, regardless of incomplete reactions or other defaults. Beyerman *et al.* (1973a) pointed out that this type of operation cannot be termed "automated" because this would require monitoring for completeness of reactions connected with feedback control. To date, none of the presently known programmed electromechanical instruments possesses these features. Several types of modified machines have been developed. The main concerns in these designs have been (a) the ways and means to deliver fault-free a multitude of solvents and reagents in measured quantities into the reaction vessel and (b) the type of programming. Brunfeldt, Halstrøm, and Roepstorff (1968, 1969a; Brunfeldt, 1969) designed an instrument aimed at improving delivery of liquids with minimal risk of contamination and wide flexibility of programming. Solvents and reagent solutions are transferred by gas pressure through individual separate tubings. In this way liquid distributor valves are avoided with their inherent problems of leakage, clogging, or contamination. The programming information is supplied through a punched tape, produced by an eight-channel teletype printer. Instrumentation for computer generated paper tape programs attached to a mechanized synthesizer has been described by Tometsko *et al.* (1971).

An instrument built by Loffet and Close (1968) basically resembles the layout of Merrifield's machine in using a distributor valve for reagent solutions. A disk programmer is used, and the schedule of Merrifield *et al.* (1966) for carbodiimide coupling has been employed for synthesis.

Mansveld, Hindriks, and Beyerman (1968) use in their instrument "a mechanical part which is similar to that of Merrifield." To permit flexible programming of a wide variety of synthetic methods, of reaction and agitation times, of solvents and reagents, a disk programmer has been chosen. This programmer is operated through an x-y matrix plug board with horizontal and vertical bars to be interconnected by simple plugging. Efforts are presently being made to fully automate this instrument by installing a feedback control with the use of [^{14}C]-labeled *tert*-butoxycarbonyl protecting groups (Beyerman *et al.*, 1973a).

An instrument described by Hruby *et al.* (1972) features a dual programming scheme. The variable operations are controlled by a drum programmer, for example, selection of type and amounts of solvents or reagents. The repetitive operations needed to introduce and remove solvents and reagents are controlled by a card programmer. Fail-safe devices, e.g., a postmetering vessel, are built in for shut-off in case of failures. The operation is monitored by a strip chart recorder which gives a permanent record of a synthesis. Another machine has recently been designed by Boni *et al.* (1971).

Commercially available machines include those by Schwarz/Mann Company, Orangeburg, New York (Brunfeldt design); by Beckman-Spinco Instruments, Inc., Palo Alto, California (Merrifield design); by Vega Engineering Company, Tuscon, Arizona; by Shimadzu Company, Japan; and by Chemtrox Corporation, Rochester, New York (Tometsko design).

These instruments certainly provide additional savings in time and labor. Overnight and weekend operation is provided and up to six cycles per day can be carried out when carbodiimide is used for coupling, or up to two cycles per day when the active ester method is used. In reality, the requirements for longer reaction times (see Section III,D,1) and for analytic controls (see Section IV,D) restrict the number of cycles that can be completed in a day (except for routine preparations of known peptides using fully explored systems). It should be very interesting to see to what extent feedback-controlled automation will improve on the present situation. Although the instruments save time, they require, on the other hand, considerable maintenance and repair. They also need, in my opinion, a full-time technically versed operator. It is therefore difficult to assess which of the two alternatives is more effective, simple manually operated shaker or programmed mechanized instrument. Again, as with many other aspects of solid-phase synthesis, it depends on the peptides to be synthesized.

In the light of this situation several simple mechanized instruments without programmers appear to be attractive alternatives. Two models by Samuels and Holybee have been described and illustrated by Stewart and Young (1969). Young *et al.* (1972) developed another instrument of this type, called the "Peptider."* Similar assemblies of bottles, tubes, valves, and reaction vessel are probably in use in many laboratories and might at present best meet the overall equipment and operational demands of the solid-phase method.

V. Applications

Through nearly one decade the solid-phase method has been applied to syntheses of peptides of all known categories. Some groups of peptides have given comparatively better results than others which have proven difficult through repeated attempts. In the early period bradykinin was found to be readily prepared by solid-phase synthesis and angiotensin syntheses also gave very good results. More recently many analogs of oxytocin and vasopressin have been prepared. For syntheses of gramicidin S and antamanide,

* Commercially available from Peninsula Laboratories, 1105 Laurel Street, San Carlos, Cal. 94070.

the solid-phase method appears to be ideally suited. In his recent review Merrifield (1969a) has described in detail the advantages, the difficulties, and the peculiarities that have arisen during solid-phase preparations of bradykinin, angiotensin, oxytocin and vasopressin, insulin (see, also, Lübke and Klostermeyer, 1970), ferredoxin, synthetic antigens, nylon oligomers, and depsipeptides.

Several tables have been published listing peptides prepared by solid-phase procedures (Merrifield, 1968a, 1969a). Marshall and Merrifield (1970) listed all syntheses published from the beginning until October 1969, in the second edition of the CRC Handbook of Biochemistry. In these tables all peptides have been arranged by their size. Reaction conditions, α -amino protecting groups used and purification procedures are given for each synthesis. The review of Losse and Neubert (1970a) contains a table listing peptides prepared by solid-phase synthesis up to early 1969. This table also records $N\alpha$ - and side-chain protecting groups, reagents, solvents, and ratios as well as reaction times used. The arrangement is by year of publication with an approximately chronological order within each year-group.

The material listed in the above-mentioned tables has been included here. The following tables are strictly a literature source with no synthetic data included. The arrangement of peptides is by functional or generic relation. Model peptides are listed separately from those related to naturally occurring biologically active peptides. The latter are grouped broadly on the basis of their biological function and appear in alphabetical order of their names within each group. Literature has been covered until summer, 1971.

A. MODEL PEPTIDES

A large number of peptides have been prepared as models for the purpose of testing the performance of solid-phase systems. Merrifield's first reports on the new method (1962, 1963) described the synthesis of the model tetrapeptide Leu-Ala-Gly-Val, which was obtained in crystalline form. This same model peptide has since been used many times as a test system. Numerous other model peptides ranging from dipeptides to a polymer containing 133 amino acid residues have been synthesized. These are listed in the order of size in Table XXII.

B. PEPTIDES WITH BIOLOGICAL FUNCTIONS

1. *Hormones*

Peptide hormones prepared by solid-phase synthesis cover the widest range in size. The smallest solid-phase synthetic hormone is the tripeptide

Table XXII—Peptides (Models) Prepared by Solid-Phase Synthesis

Dipeptides	
Ala-Ala	Beyerman <i>et al.</i> (1973a)
L-Ala-Ala-L	Fridkin <i>et al.</i> (1965a,b); Patchornik <i>et al.</i> (1967)
Boc-Asn-Ala-OMe	Pereira <i>et al.</i> (1969b)
Boc-Asp(OBzl)-Ala-OBzl	Laufer <i>et al.</i> (1968)
Boc-Asp(OMe)-Ala-OMe	Pereira <i>et al.</i> (1969b)
Boc-Met-Ala-OMe	Pereira <i>et al.</i> (1969b)
Boc-Thr-Ala-OBzl	Laufer <i>et al.</i> (1968)
Gly-Ala	Beyerman <i>et al.</i> (1973a)
Pht-Gly-Ala-OEt	Sklyarov <i>et al.</i> (1966)
Z-Gly-D-Ala-OEt	Sklyarov <i>et al.</i> (1966)
Gly-DL-Ala	Vlasov and Bilbin (1969)
Z-Gly-DL-Ala-OBzl	Sklyarov <i>et al.</i> (1966)
Boc-Pro-Ala-OMe	Halpern <i>et al.</i> (1968)
Boc-Trp-Ala-OMe	Halpern <i>et al.</i> (1968)
Dim-Val-Ala	D��r (1966)
Dim-Val-Ala-OMe	Pereira <i>et al.</i> (1969b)
Thr-Arg	Young <i>et al.</i> (1967)
[¹⁴ C]-Oct-Thr-Arg	Benjamini <i>et al.</i> (1968b)
Nps-Gly-Asn-OMe	Beyerman (1972)
Ala-Cys(Bzl)	Beyerman <i>et al.</i> (1973a)
Gly-Cys(Bzl)	Beyerman <i>et al.</i> (1973b)
Nps-Gly-Cys(Bzl)-OMe	Beyerman (1972)
Pht-Gly-Glu(OBzl) ₂	Wolman <i>et al.</i> (1967)
His-Glu	Windridge and Jorgensen (1971a)
His(Bzl)-Glu	Windridge and Jorgensen (1971b)
Ala-Gln	Dorman <i>et al.</i> (1972)
Ala-Gly	Beyerman <i>et al.</i> (1973a)

- [Ala-Gly]
 Z-Ala-Gly-OEt
 Z-Dl-Ala-Gly-OEt
 Boc-Ala-Gly-OPic
 Cys-Gly
 Z-Cys(Z)-Gly-OBzl
 Nps-Cys(Trt)-Gly-OMe
 Gln-Gly-OEt
 Gly-Gly
 [Gly-Gly]
 Z-Gly-Gly-OEt
 Nps-Gly-Gly-OMe
 Leu-Gly
- Boc-Lys(Z)-Gly-OMe
 [Phe-Gly]
 Boc-Phe-Gly-OEt (-NHBzl)
 Nps-Phe-Gly-OMe
 Z-Pro-Gly-OBzl
 <Glu-Gly-OEt
 Z-Tyr(Z)-Gly-OEt
 Boc-Tyr(Bzl)-Gly-OMe
 Boc-Gln-His(Bzl)-OBzl
 Nps-Gly-Leu-OMe
 Leu-Leu
 Z-Phe-Leu-OMe
 Boc-Pro-Leu-OMe
 Ala-Phe
 Asn-Phe
- Fridkin *et al.* (1965a,b); Patchornik *et al.* (1967); Flanigan and Marshall (1970, 1972)
 Fridkin *et al.* (1965b)
 Manecke and Haake (1968)
 Camble *et al.* (1969)
 Lunkenheimer and Zahn (1970b)
 Fridkin *et al.* (1966a); Wolman *et al.* (1967)
 Beyerman (1972)
 Beyerman *et al.* (1973b)
 Beyerman *et al.* (1973a); Steinman (1967a)
 Fridkin *et al.* (1965a,b); Patchornik *et al.* (1967); Flanigan and Marshall (1970, 1972)
 Wolman *et al.* (1967)
 Beyerman (1972)
 Letsinger and Kornet (1963); Letsinger *et al.* (1964); Felix and Merrifield (1970); Mukaiyama *et al.* (1970); Maher *et al.* (1971)
 Pereira *et al.* (1969b)
 Fridkin *et al.* (1965a,b); Patchornik *et al.* (1967)
 Wieland *et al.* (1970)
 Beyerman (1972)
 Fridkin *et al.* (1966a)
 Beyerman *et al.* (1973b)
 Fridkin *et al.* (1965b)
 Pereira *et al.* (1969b)
 Laufer *et al.* (1968)
 Beyerman (1972)
 Steinman (1967b)
 Fridkin *et al.* (1965b)
 Halpern *et al.* (1968)
 Tilak and Hollinden (1968a); Beyerman *et al.* (1973a)
 Sano *et al.* (1971)

Table XXII, *cont.*

Dipeptides, <i>cont.</i>	
Boc-Ala-Phe-OMe	Wieland and Birr (1966)
Boc-Asp(OBzl)-Phe-OMtp	Flanigan and Marshall (1972)
Gly-Phe	Beyerman <i>et al.</i> (1973a)
[<u>Gly-Phe</u>]	Flanigan and Marshall (1970, 1972)
Boc-Gly-Phe-OMe	Wieland and Birr (1966)
Boc-His(Bzl)-Phe-OMe	Pereira <i>et al.</i> (1969b)
Ile-Phe	Sano <i>et al.</i> (1971)
Boc-Leu-Phe-OMe	Wieland and Birr (1966)
Boc-Pro-Phe-OMe	Halpern <i>et al.</i> (1968)
Boc-Pro-Phe-OPic	Camble <i>et al.</i> (1969)
Trp-Phe	Marshall (1968)
Tyr-Phe	Arai <i>et al.</i> (1970)
Boc-Val-Phe-OMe	Halpern <i>et al.</i> (1968)
Ala-Pro	Beyerman <i>et al.</i> (1973a)
Gly-Pro	Beyerman <i>et al.</i> (1973a)
Nps-Phe-Pro-OMe	Beyerman (1972)
Boc-Pro-Val-OMe	Halpern <i>et al.</i> (1968)
Val-Val	Weygand and Ragnarsson (1966)
Tripeptides	
Ac-Lys(Ac)-D-Ala-D-Ala	Perkins and Nieto (1970)
Cys-Cys-Ala	Patchornik (1967); Patchornik <i>et al.</i> (1967); Bondi <i>et al.</i> (1968)
Boc-Leu-Cys(Bzl)-Ala-OMe	Pereira <i>et al.</i> (1969b)
Boc-Ala-Glu(OMe)-Ala-OMe	Pereira <i>et al.</i> (1969b)
Ile-Ile-Ala	Geising and Hörnle (1973)
Dim-Val-Leu-Ala	Déer (1966)

Gly-Phe-Ala	Semkin <i>et al.</i> (1967a)
Z-Gly-Phe-Ala-OMe	Sklyarov <i>et al.</i> (1966)
Z-Phe-Phe-Ala-ONb	Fridkin <i>et al.</i> (1966a)
Boc-Leu-Tyr-Ala-NH ₂	Ohno and Anfinson (1967)
Arg-Gly-Arg	Abramson <i>et al.</i> (1970)
Ala-Thr-Arg	Young <i>et al.</i> (1967)
[¹⁴ C]-Oct-Ala-Abu-Arg	Benjamini <i>et al.</i> (1969)
[¹⁴ C]-Oct-Abu-Abu-Arg	Benjamini <i>et al.</i> (1969)
[¹⁴ C]-Oct-Ala-Gly-Arg	Benjamini <i>et al.</i> (1969)
[¹⁴ C]-Oct-Ala-Leu-Arg	Benjamini <i>et al.</i> (1969)
[¹⁴ C]-Oct-Ala-Ser-Arg	Benjamini <i>et al.</i> (1969)
Oct-Ala-Thr-Arg	Benjamini <i>et al.</i> (1968b)
[¹⁴ C]-Oct-Ala-Thr-Arg	Benjamini <i>et al.</i> (1969)
[¹⁴ C]-Oct-Abu-Thr-Arg	Benjamini <i>et al.</i> (1969)
[¹⁴ C]-Oct-Gly-Thr-Arg	Benjamini <i>et al.</i> (1969)
[¹⁴ C]-Oct-Leu-Thr-Arg	Benjamini <i>et al.</i> (1969)
Boc-Val-Gly-Asp(OMe)-OMe	Pereira <i>et al.</i> (1969b)
Dimoz-Phe-Ala-Gly	Wieland and Birr (1967a)
Gly-Gly-Gly	Mitchell and Roeske (1970)
Phe-Gly-Gly	Smith and Dunn (1966)
Pro-Leu-Gly	Loffet <i>et al.</i> (1971)
Z-Phe-Lys(Boc)-Gly-NH ₂	Kessler and Iselin (1966)
Tyr-Phe-Leu	Arai <i>et al.</i> (1970)
Phe-Ala-Phe-NH ₂	Pietta and Marshall (1970)
Pro-Ala-Phe	Wieland and Racky (1968)
Boc-Ala-Leu-Phe-OMe	Pereira <i>et al.</i> (1969b)
Z-Phe-Phe-Phe-NH ₂	Fridkin <i>et al.</i> (1966a)
His(Bzl)-Pro-Phe	Windridge and Jorgensen (1971b)
Ala-Ser-Phe	Polzhofer (1969b)
Ala-Thr-Phe	Polzhofer (1969b)
Z-Gly-Val-Phe	Wieland and Birr (1966)

Table XXII, *cont.*

Tripeptides, <i>cont.</i>	
Boc-Tyr(Bzl)-Val-Phe-N ₃ H ₈	Flanigan and Marshall (1972)
Ac-Val-Met-Pro-OMe	Roepstorff <i>et al.</i> (1970)
Boc-Phe-Ala-Ser(Bzl)-OMe	Pereira <i>et al.</i> (1969b)
Z-Gly-Val-Thr(Bzl)-OMe	Pereira <i>et al.</i> (1969b)
Z-Gly-Gly-Tyr-OBzl	Fridkin <i>et al.</i> (1965b)
Ala-Ala-Val	Beyerman <i>et al.</i> (1973a)
Asp-Ala-Val	Marshall and Merrifield (1965)
Gly-Ala-Val	Beyerman <i>et al.</i> (1973a)
Leu-Ala-Val	Esko and Karlsson (1970); Dorman <i>et al.</i> (1971)
Ala-Gly-Val	Khosla <i>et al.</i> (1972)
Leu-Gly-Val	Dorman <i>et al.</i> (1971)
Leu-Phe-Val	Weygand and Ragnarsson (1966)
Tetrapeptides	
[Ala-Ala-Ala-Ala-]	Fridkin <i>et al.</i> (1965a,b); Patchornik <i>et al.</i> (1967)
Z-Ala-Ala-Ala-Ala-ONb	Fridkin <i>et al.</i> (1965b)
Boc-Thr-Ala-Ala-Ala-OBzl	Laufer <i>et al.</i> (1968)
[Ala-Gly-Ala-Ala-]	Fridkin <i>et al.</i> (1965a,b); Patchornik <i>et al.</i> (1967)
Z-Leu-Phe-Ala-Ala	Weygand (1968)
Phe-Gly-Leu-Ala	Brunfeldt <i>et al.</i> (1969b, 1971)
Pro-Gly-Phe-Ala	Parr and Grohmann (1971)
Boc-Trp-Phe-Pro-Ala-OMe	Pereira <i>et al.</i> (1969b)
<Glu-Phe-Ala-Arg	Lenard and Robinson (1967)
Arg-Gly-Gly-Arg	Abramson <i>et al.</i> (1970)
Asp-Ala-Thr-Arg	Young <i>et al.</i> (1967)
¹⁴ C]-Oct-Asp-Ala-Thr-Arg	Benjamini <i>et al.</i> (1968b)

-Ala-Ala-Ala-Gly-
 Z-Ala-Leu-Ala-Gly
 [α, α^3 H₂]-Gly-Gly-Gly
 Gly-Gly-Gly-[α, α^3 H₂]-Gly
 Gly-Gly-Leu-Gly
 Ac-Thr-Gly-Lys-Gly
 Ac-Thr-Trp-Lys-Gly
 Ala-Leu-Phe-Gly
 Boc-Ala-Leu-D-Phe-Gly-OBzl
 Aoc-Phe-Phe-Phe-Gly-N₂H₅
 Gly-Asp-Ser-Gly
 Z-Phe-Val-Ala-Leu-N₂H₅
 Ac-Ile-Gly-Leu-Met-OMe
 Boc-Pro-Pro-Ala-Phe
 Dim-Leu-Val-Ala-Phe
 Trp-Met-Asp-Phe-NH₂
 All DL-Leu-Ala-Gly-Phe
 All DL-Leu-Leu-Gly-Phe
 Boc-Leu-Leu-Val-Phe-NH₂
 Leu-Leu-Leu-Tyr
 Boc-Leu-Leu-Leu-Tyr(Bzl)-OMe
 Leu-Asp-Ala-Val
 Ile-Leu-Ala-Val
 Leu-Ala-Gly-Val
 Z-Leu-Ala-Gly-Val-N₂H₅
 Z-Leu-Ala-Gly-Val-OPic
 Leu-Ala-Gly-Val
 Ile-Ile-Leu-Val

Table XXII, *cont.*

Pentapeptides	
Tyr-D-Glu-Lys-D-Ala-D-Ala	Perkins and Nieto (1970)
Boc-Met-Phe-Gly-Leu-Ala-OMe	Pereira <i>et al.</i> (1969b)
Dim-Val-Val-Phe-Leu-Ala	D��r (1966)
Ala-Ile-Arg(NO ₂)-Ser-Ala	Green and Garson (1969)
Dns-Pro-Leu-Gly-Pro-Arg	Sch��llman (1967)
Gly*-Gly-Thr-Asn-Glu	Robinson <i>et al.</i> (1970)
Gly*-Ala-Asn-Ala-Gly	Robinson <i>et al.</i> (1970)
Gly*-Arg-Asn-Arg-Gly	Robinson <i>et al.</i> (1970)
Gly*-Glu-Asn-Glu-Gly	Robinson <i>et al.</i> (1970)
Gly*-Thr-Asn-Glu-Gly	Robinson <i>et al.</i> (1970)
Phe-Gly-Gly-Gly-Gly	Smith and Dunn (1970)
Phe-Gly-Gly-Gly*-Gly	Smith <i>et al.</i> (1970)
Thi-Gly-Gly-Gly-Gly	Smith and Dunn (1970)
Gly*-Lys-Asn-Lys-Gly	Robinson <i>et al.</i> (1970)
Leu-Ala-Val-Phe-Gly	Vlasov and Bilbin (1969)
Gly*-Thr-Asn-Thr-Gly	Robinson <i>et al.</i> (1970)
Boc-Gly-Ala-Phe-Val-Gly	Shchukina and Sklyarov (1966)
Ala-Val-Val-Val-Gly	Gut and Rudinger (1968)
Pro-Ala-Phe-Phe-Leu	Beyerman <i>et al.</i> (1967b)
Tyr-Leu-Gly-Gly-Phe	Wieland <i>et al.</i> (1969b)
All DL-Phe-Leu-Leu-Gly-Phe	Losse <i>et al.</i> (1968a)
Boc-Ala-Ala-Ala-Lys(Z)-Phe-OMe	Laufer <i>et al.</i> (1968)
Ala-Val-Val-Val-Phe	Gut and Rudinger (1968)
Sar-Nle-Phe-Ala-Pro	Sipos and Gaston (1971)
Sar-Lys-Trp-Ala-Pro	Sipos and Gaston (1971)
<Glu-Arg(NO ₂)-Phe-Leu-Pro	Sipos and Gaston (1971)
Leu-Ile-Ile-Leu-Val	Weygand and Obermeier (1968)

Hexapeptides	
Ser-Val-Thr-Leu-Ala-Ala	Varsel <i>et al.</i> (1970)
Boc-Pro-Pro-Phe-Phe-Ala-Ala	Weygand (1968)
Gly-Asn-Gly-Met-Asn-Ala	Hruby <i>et al.</i> (1972)
Gly-Asp-Gly-Met-Asn-Ala	Hruby <i>et al.</i> (1972)
Phe-Leu-Ile-Val-Gly-Ala	Gutte and Merrifield (1971)
Val-Gly-Phe-Ala-Leu-Ala	Jakubke and Baumert (1973)
Ser-Val-Thr-Ala-Leu-Ala	Varsel <i>et al.</i> (1970)
Boc-Gly-Lys(Z)-Phe-Gly-Leu-Ala-OMe	Pereira <i>et al.</i> (1969b)
Leu-Leu-Leu-Leu-Ala	Tometsko <i>et al.</i> (1970)
Dim-Val-Gly-Val-Phe-Leu-Ala	D��r (1966)
Ser-Val-Ala-Thr-Leu-Ala	Varsel <i>et al.</i> (1970)
Boc-Leu-Ala-Tyr-Ile-Tyr-Ala-N ₂ H ₅	Ohno and Anfinsen (1967)
Boc-Leu-Ala-Tyr(Bzl)-Ile-Tyr(Bzl)-Ala-N ₂ H ₅	Anfinsen (1968)
[Gly-Val-Ala-Phe-Ala-Gly]	Flanigan and Marshall (1970, 1972)
Gly-Lys-Asn-Lys-Arg-Gly	Lenard and Robinson (1967)
[Val-Ala-Phe-Ala-Gly-Gly]	Wieland <i>et al.</i> (1970)
Boc-Val-Ala-Phe-Ala-Gly-Gly-N ₂ H ₅ - 	Wieland <i>et al.</i> (1970)
Ac-Ala-Met-Ala-Leu-Phe-Gly-OMe	Roepstorff <i>et al.</i> (1970)
Ala-Gly-Gly-Leu-Phe-Gly	Sano <i>et al.</i> (1971)
Leu-Phe-Gly-Leu-Phe-Gly	Sano <i>et al.</i> (1971)
Val-Val-Thr-Ala-Ala-His	Stewart <i>et al.</i> (1967)
Ala-Phe-Ile-Gly-Leu-Met	Brunfeldt <i>et al.</i> (1971)
Ala-Phe-Pro-Leu-Glu-Phe	Kaiser <i>et al.</i> (1970)
All D-Leu-Leu-Phe-Leu-Gly-Phe	Losse <i>et al.</i> (1968a)
Glu-Lys-Lys-Ser-Leu-Pro	Omenn and Anfinsen (1968)
Phe-Tyr-Ile-Ile-Ala-Val	Esko and Karlsson (1970)
Leu-Leu-Gln-Gly-Leu-Val-OMe	Bodanszky and Sheehan (1966)

Table XXII, *cont.*

Heptapeptides	
Gly-Pab-Gly-His(Bzl)-Gly-Pab-Acp	A. R. Mitchell <i>et al.</i> (1970)
Trp-Trp-Cys(CM)-Asn-Asp-Gly-Arg	Hruby <i>et al.</i> (1972)
Gly-Pro-Gly-Phe-Gly-Pro-Gly	Sugiyama <i>et al.</i> (1968)
Val-Val-Thr(Val)-Ala-Ala-His	Stewart <i>et al.</i> (1967)
Lys, $n = 2-7$	Anfinsen (1968)
Ala-Arg-Arg-Leu-Arg-Arg-Phe	Yamashiro <i>et al.</i> (1972)
Adenyl ^{yl} } Uridyl ^{yl} } -(5'→N)-Gly-Ala-Gly-Ala-Gly-Leu-Phe	Smirnov <i>et al.</i> (1969)
Octapeptides	
Boc-Met-Asp(OBzl)-Ser(Bzl)-Ser(Bzl)-Thr-Ser(Bzl)-Ala-Ala-OBzl	Laufer <i>et al.</i> (1968)
Pro-Gly-Thr-Lys-Met-Ile-Phe-Ala	Nanzyo and Sano (1968)
Gly-Gly-Gly-Val-Arg-Gly-Pro-Ala-NH ₂	Andreatta <i>et al.</i> (1971)
Acp, $n = 4,8$	Kusch (1966a,b)
Gly-Pro-Gly-Pro-Pro-Gly-Ala-Lys	Kettman <i>et al.</i> (1967)
Leu-Ala-Tyr-Lys-Lys-Lys-Lys	Omenn and Anfinsen (1968)
All DL-Phe-Phe-Gly-Leu-Leu-Gly-Phe	Losse <i>et al.</i> (1968b)
Nonapeptides	
Dnp-Lys ₉	Yaron and Schlossman (1966, 1968)
Dnp-Lys-D-Lys-Lys ₄	Yaron and Schlossman (1966, 1968)
Dnp-Lys-D-Lys-Lys ₇	Schlossman <i>et al.</i> (1969)
Dnp-Lys-D-Lys-Lys	Schlossman <i>et al.</i> (1969)
Dns-(Pro) _n $n = 2-9$	Gabor (1968)

Decapeptides		
Ile-Val-Leu-Ile-Phe-Gly-Ile-Val-Leu-Ala Aud, $n = 3,4,5,10$ Lyso		Geising and Hörnle (1973) Kusch (1966a,b) Grahl-Nielsen and Tritsch (1969) Kusch (1966a,b)
Oligo-Hexamethylenadipinamide, Nylon 6.6 oligomers (-NH(CH ₂) ₆ NH-CO(CH ₂) ₆ CO-) _n $n = 2,3,4,5,10$		
Number of residues	Peptides with more than Ten Amino Acid Residues	
11	Ile-Val-Cys(Bzl)-Glu(OBzl)-Ala-Phe-Gly-Ile-Val-Leu-Ala (Leu-Ala) ₆	Geising and Hörnle (1973)
12	Ile-Val-Leu-Cys(Bzl)-Glu(OBzl)-Ala-Phe-Gly-Ile-Val-Leu-Ala (Ala-Phe) ₆ Dns-(Pro) _n -N ₂ H ₃ $n = 2-12$ (-Pro-Gly-Pro) _n $n = 1-4$ (-Pro-Gly-FPro) _n $n = 1-4$ (-Gly-Pro-MePro) _n $n = 1-4$ Leu-Leu-Leu-Val-Asp-Thr-Pro-Glu-Thr-Lys-His-Pro-Lys	Hagenmaier (1970); Bayer and Koenig (1969); Bayer (1970); Bayer <i>et al.</i> (1971b) Geising and Hörnle (1973) Bayer (1970) Stryer and Haugland (1967) Hutton <i>et al.</i> (1968) Hutton <i>et al.</i> (1968) Hutton <i>et al.</i> (1968) Omenn and Anfinsen (1968)
13		
14	Gly-Ser-Thr-Asp-Tyr-Gly-Ile-Leu-Gln-Ile-Asn-Ser-Arg-Gly	Hruby <i>et al.</i> (1972)
15	Cys-Ala-Val-Cys-His(Bzl)-Ala-Val-Ala-Lys-Ala-Ala-Ala-Ala-Ala	Lenard and Robinson (1967)
16	Tyr-His-His-Phe-Phe-Asp-His-Ser-Asp-Ser-His-Phe-Asp-Phe-His-Phe	Woolley (1966)
17	Met-Thr-Phe-Arg-Leu-Leu-Val-Asp-Thr-Pro-Glu-Thr-Lys-His-Pro-Lys	Omenn and Anfinsen (1968)

Table XXII, *cont.*

Number of residues	Peptides with more than Ten Amino Acid Residues, <i>cont.</i>
20	Pro _n $n = 2-17$ Rothé <i>et al.</i> (1969, 1971)
24	(Ile-Val-Leu-Ala) ₈ Geising and Hörnle (1973)
25	(-Pro-Gly-Gly-) _n $n = 1-8$ Rothé <i>et al.</i> (1968, 1969)
	(Ala-dL) ₂₄ -Gly-d ₃ Tripp (1968)
	Acp _n $n = \text{up to } 25$ Rothé <i>et al.</i> (1966, 1968); Rothé and Schneider (1966); Rothé and Dunkel (1967)
50	DL-Lys ₁₈ -Val ₁₀ -DL-Lys ₁₀ -Gly Epand and Scheraga (1968)
60	(-Pro-Pro-Gly-) _n $n = 10, 20$ Sakakibara <i>et al.</i> (1968)
71	(DL-Lys) ₆₀ -Leu ₁₀ -Gly Ostroy <i>et al.</i> (1970)
133	(DL-Lys) ₇₁ -Leu ₂₁ -(DL-Lys) ₈₇ -Gly Ostroy <i>et al.</i> (1970)
—	DL-Lys _m -Ala _n -DL-Lys _m Ingwall <i>et al.</i> (1968)

thyrotropin releasing hormone; the largest is a protein preparation synthesized according to a target sequence of human growth hormone (188 amino acid residues). The method has been most frequently applied in syntheses of insulin chains and analogs (approximately fifty products) and in preparations of over forty neurohypophyseal hormones (oxytocin, vasopressin, and analogs). Table XXIII lists hormone syntheses: (A) insulin chains, (B) neurohypophyseal hormones, and (C) other peptide hormones.

2. Kinins

A very successful field of modern integrated peptide research comprising physiology, pharmacology, and physical chemistry is that of angiotensin. Solid-phase synthesis contributed in a major way to an understanding of angiotensin action. Approximately 80 angiotensins and analogs have been synthesized for the study of structure-activity correlations. Another intensively investigated area is that of bradykinin, and approximately sixty analogs have been prepared by solid-phase synthesis. Table XXIV lists solid-phase synthetic kinins: (A) angiotensin, and (B) bradykinin, eledoisin, and physalaemin.

3. Other Biologically Active Peptides and Proteins

Table XXV is subdivided in (A) antibiotics, toxins, and related peptides, (B) enzymes, and (C) other biologically active peptides. The cyclic decapeptides, gramicidin S, and antamanide, have been obtained in yields and degrees of homogeneity that attest to almost ideal courses of solid-phase synthesis in these systems. In the enzyme area the ribonuclease A, staphylococcal nuclease P₂ peptide, and nuclease T₁ preparations are outstanding examples among many other applications. Encephalytogenic protein from myelin has been an object of intensive study. A search for antigenic sites in tobacco mosaic virus protein has also been an incentive for solid-phase synthesis of a number of peptides with TMV-protein sequences. These examples point only to a few of the many kinds of peptides that have been prepared.

C. SPECIAL APPLICATIONS OF THE SOLID-PHASE METHOD

By its unique design solid-phase synthesis is ideally suited for several particularly specialized applications. The insolubility of the growing peptide chain and the fact that it is retained at all times in the same vessel make the procedure the most advantageous for the *preparation of internally radioactively labeled peptides*. The purification by washing and filtration rather than by precipitation or crystallization, as used in solution synthesis, further minimizes the hazards involved with the handling of radioactive products.

Table XXIII—Peptide and Protein Hormones Prepared by Solid-Phase Synthesis

A. Insulin Chains		Insulin A-chains	
Gly-Ile-Val-Glu-Gln-Cys-Cys-Ala-Ser-Val-Cys-Ser-Leu-Tyr-Gln-Leu-Glu-Asn-Tyr-Cys-Asn (bovine)		5	20
-Thr-Ser-Ile-		10 human, porcine	15
-Ala-Gly-Val-		10 ovine	20
Insulin A-chain, bovine			
[Ala ¹]-Insulin A-chain, bovine			Marglin and Merrifield (1966); Marglin (1969); Hörnle (1967); Zahn <i>et al.</i> (1967a,b)
[Ala ²]-Insulin A-chain, ovine			Weber <i>et al.</i> (1967); Weitzel <i>et al.</i> (1968a,b)
[Leu ³]-Insulin A-chain, ovine			Weber <i>et al.</i> (1967); Weitzel <i>et al.</i> (1968a,b)
[Val ⁴]-Insulin A-chain, ovine			Hörnle <i>et al.</i> (1968)
[Ala ¹²]-Insulin A-chain, ovine			Hörnle <i>et al.</i> (1968)
[Ala ⁶ , Ala ¹²]-Insulin A-chain, ovine			Weitzel <i>et al.</i> (1968a,b); Weber (1969); Weber <i>et al.</i> (1971)
[Ala ¹² , Ala ¹⁷]-Insulin A-chain, ovine			Weber <i>et al.</i> (1968)
[Ala ¹² , Ala ¹⁷]-Insulin A-chain, ovine			Weitzel <i>et al.</i> (1968a,b)
[Ala ¹² , Ala ¹⁷]-Insulin A-chain, ovine			Weitzel <i>et al.</i> (1968a,b)
[Ala ¹² , Phe ¹⁹]-Insulin A-chain, ovine			Weitzel <i>et al.</i> (1968a,b)
[β-Ala ¹ , Ala ¹⁷]-Insulin A-chain, ovine			Weitzel <i>et al.</i> (1968a,b)
[Ala ² , Ala ¹⁷]-Insulin A-chain, ovine			Weitzel <i>et al.</i> (1968a,b)
[Ala ⁶ , Ala ¹⁷]-Insulin A-chain, ovine			Weitzel <i>et al.</i> (1968a,b)
[Ala ⁶ , Ala ¹⁷]-Insulin A-chain, ovine			Weitzel <i>et al.</i> (1968a,b)
[Leu ¹⁰ , Ala ¹⁷]-Insulin A-chain, ovine			Weitzel <i>et al.</i> (1968a,b)
[Leu ³ , Ala ¹⁷]-Insulin A-chain, ovine			Weitzel <i>et al.</i> (1968a,b)
[Ile ⁹ , Ala ¹⁷]-Insulin A-chain, ovine			Weitzel <i>et al.</i> (1968a,b)
[Ala ¹² , Phe ¹⁴ , Phe ¹⁹]-Insulin A-chain, ovine			Weitzel <i>et al.</i> (1968a,b)

[Ala ¹³ , Phe ¹⁹ , Ala ²¹]-Insulin A-chain, ovine	Weitzel <i>et al.</i> (1968a,b)
[Ala ¹³ , Ala ¹⁶ , Phe ¹⁹ , Ala ²¹]-Insulin A-chain, ovine	Weitzel <i>et al.</i> (1968a,b)
[Leu ³ , Leu ⁸ , Leu ¹⁰ , Ala ¹⁷]-Insulin A-chain, ovine	Weitzel <i>et al.</i> (1968a,b)
Des-Gly ¹ -[Ala ¹⁷]-Insulin A-chain, ovine	Weitzel <i>et al.</i> (1968a,b)
Des-Gly ¹ -[Gly ²]-Insulin A-chain, bovine	Weber <i>et al.</i> (1967); Weitzel <i>et al.</i> (1968a,b)
[Ala ⁷ , Ala ²⁰]-Insulin A-chain-(6-11)disulfide, bovine	Marglin and Cushman (1967); Marglin (1969)
[Glu ⁵ , Ala ¹² , Ala ¹⁹ , Ala ²¹]-Insulin A-chain, ovine	Weber <i>et al.</i> (1971)
[Glu ⁵ , Ala ¹² , Glu ¹⁵ , Ala ¹⁸ , Phe ¹⁹ , Ala ²¹]-Insulin A-chain, ovine	Weber <i>et al.</i> (1969)
[Glu ⁵ , Ala ¹⁰ , Ala ¹² , Glu ¹⁵ , Ala ¹⁸ , Phe ¹⁹ , Ala ²¹]-Insulin A-chain	Weber <i>et al.</i> (1969)
[Glu ⁵ , Ala ⁴ , Ala ¹⁴ , Glu ¹⁶ , Ala ¹⁸ , Phe ¹⁹ , Ala ²¹]-Insulin A-chain, ovine	Hörnle <i>et al.</i> (1968); Weber <i>et al.</i> (1969)
[Val ¹² , Glu ⁵ , Ala ¹⁹ , Ala ¹⁴ , Glu ¹⁵ , Ala ¹⁸ , Phe ¹⁹ , Ala ²¹]-Insulin A-chain, ovine	Hörnle <i>et al.</i> (1968)
[Glu ⁵ , Ala ¹⁰ , Ala ¹² , Ala ¹⁴ , Glu ¹⁶ , Ala ¹⁸ , Phe ¹⁹ , Ala ²¹]-Insulin A-chain	Weber <i>et al.</i> (1969)
[Glu ⁵ , Ala ¹² , Ala ¹³ , Ala ¹⁴ , Glu ¹⁵ , Ala ¹⁸ , Phe ¹⁹ , Ala ²¹]-Insulin A-chain	Weber <i>et al.</i> (1969)
ovine	
Insulin B-chains	
Phe-Val-Asn-Gly-His-Leu-Cys-Gly-Ser-His-Leu-Val-Glu-Ala-Leu-Tyr-Leu-Val-Cys-Gly-Glu-Arg-Gly-Phe-	
5 10 15 20	
Phe-Tyr-Thr-Pro-Lys-Ala (bovine, ovine)	
25 30	
-Thr (human)	
30	
Insulin B-chain, bovine, ovine	Marglin and Merrifield (1966); Merrifield and Marglin (1967); Zahn <i>et al.</i> (1967a,b); Okuda and Zahn (1969)
Insulin B-chain, human	Okuda and Zahn (1969)
[Ala ²¹]-Insulin B-chain, bovine	Weber and Weitzel (1968)
[Ala ¹⁴]-Insulin B-chain, bovine	Weber and Weitzel (1968)

Table XXIII, *cont.*

Insulin B-chains, <i>cont.</i>	
[Ala ²⁷]-Insulin B-chain, bovine	Weber and Weitzel (1968)
[Ala ²⁷]-Insulin B-chain, bovine	Weitzel <i>et al.</i> (1969)
[Ala ²⁸]-Insulin B-chain, bovine	Weitzel <i>et al.</i> (1969)
[Ala ²⁹]-Insulin B-chain, bovine	Weitzel <i>et al.</i> (1969)
[Ala ⁶ , Ala ²⁸]-Insulin B-chain, bovine	Weitzel <i>et al.</i> (1970)
[Ala ¹⁰ , Ala ²⁸]-Insulin B-chain, bovine	Weitzel <i>et al.</i> (1970)
[Ala ⁹ , Ala ²⁷ , Ala ²⁸]-Insulin B-chain, bovine	Weitzel <i>et al.</i> (1969)
[Ala ⁵ , Ala ⁹ , Ala ²⁷ , Ala ²⁸]-Insulin B-chain, bovine	Weitzel <i>et al.</i> (1970)
[Ala ⁴ , Ala ¹⁰ , Ala ²⁷ , Ala ²⁸]-Insulin B-chain, bovine	Weitzel <i>et al.</i> (1970)
[Ala ⁶ , Ala ⁹ , Ala ¹⁰ , Ala ²⁸ , Ala ²⁹]-Insulin B-chain, bovine	Weitzel <i>et al.</i> (1970)
Des-Phe ¹ -Insulin B-chain, bovine	Weber and Weitzel (1968)
Des-(Phe ¹ , Val ²)-Insulin B-chain, bovine	Weber and Weitzel (1968)
[Ala ⁵ , Ala ⁹ , Ala ¹⁰ , Ala ²⁸ , Ala ²⁹]-Insulin B-chain-(3-30)-octacosapeptide	Weitzel <i>et al.</i> (1970)
[Ala ⁹ , Ala ²⁷]-Insulin B-chain-(1-27)-heptacosapeptide	Weitzel <i>et al.</i> (1969)
[Ala ⁴ , Ala ⁹ , Ala ²⁷]-Insulin B-chain-(1-27)-heptacosapeptide	Weitzel <i>et al.</i> (1970)
[Ala ⁴ , Ala ⁹ , Ala ²⁷ , Ala ²⁸]-Insulin B-chain-(4-30)-heptacosapeptide, bovine	Weitzel <i>et al.</i> (1969)
[Ala ⁹ , Ala ⁹ , Ala ¹⁰ , Ala ²⁷]-Insulin B-chain-(1-27)-Heptacosapeptide	Weitzel <i>et al.</i> (1970)
[Ala ⁹ , Ala ²⁷ , Ala ²⁸]-Insulin B-chain-(6-30)-pentacosapeptide, bovine	Weitzel <i>et al.</i> (1969)
[Ala ⁴ , Ala ⁹ , Ala ²⁷]-Insulin B-chain-(4-27)-tetracosapeptide	Weitzel <i>et al.</i> (1969)
[Ala ⁴ , Ala ⁵ , Ala ⁹ , Ala ²⁷]-Insulin B-chain-(4-27)-tetracosapeptide	Weitzel <i>et al.</i> (1970)
[Ala ⁴ , Ala ⁹ , Ala ¹⁰ , Ala ²⁷]-Insulin B-chain-(4-27)-tetracosapeptide	Weitzel <i>et al.</i> (1970)
[Ala ⁴ , Ala ⁵ , Ala ⁹ , Ala ¹⁰ , Ala ²⁷]-Insulin B-chain-(4-27)-tetracosapeptide	Weitzel <i>et al.</i> (1970)
[Ala ⁹ , Ala ²⁷]-Insulin B-chain-(5-27)-tricosapeptide	Weitzel <i>et al.</i> (1969)
[Ala ⁴ , Ala ⁹ , Ala ²⁸]-Insulin B-chain-(4-26)-tricosapeptide	Weitzel <i>et al.</i> (1969)

[Ala ⁵ , Ala ⁹ , Ala ¹⁰ , Ala ²⁷]-Insulin B-chain-(5-27)-tricosapeptide Insulin B-chain-(1-8)-octapeptide: Phe-Val-Asn-Gln-His-		Weitzel <i>et al.</i> (1970) Hammerström <i>et al.</i> (1970)
R Leu-Cys-Gly R = Acetyl, benzoyl, benzyl, benzyloxycarbonyl, benzylthio- methyl, diphenylmethyl, ethylcarbamoyl, p-methoxy- benzyl, tetrahydropyranyl, trityl Insulin B-chain-(24-30)-heptapeptide, human: Phe-Phe-Tyr-Thr- Pro-Lys-Thr Insulin B-chain-(24-30)-heptapeptide, bovine: Phe-Phe-Tyr-Thr- Pro-Lys-(Tos)-Ala Insulin B-chain-(14-19)-Hexapeptide amide: Z-Ala-Leu-Tyr-Leu- Val-Cys(Bzl)-NH ₂ Insulin B-chain-(27-30)-tetrapeptide, bovine: Thr-Pro-Lys-Ala Insulin B-chain-(6-8)-tripeptide: Z-Leu-Cys(Bzl)-Gly		Tilak and Hollinden (1968b) Vdovina <i>et al.</i> (1971) Ryle <i>et al.</i> (1969) Tilak and Hollinden (1968b, 1971); Marglin (1971); Vdovina <i>et al.</i> (1971) Tilak and Hollinden (1968b)
B. Neurohypophyseal Hormones		
Oxytocin		
Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly-NH ₂ 1 2 3 4 5 6 7 8 9		
Oxytocin		Beyerman <i>et al.</i> (1967b; 1968a); Bayer and Hagenmaier (1968); Bayer <i>et al.</i> (1968b); Inukai <i>et al.</i> (1968); Ives (1968); Man- ning (1968); Beyerman and In't Veld (1969); Dutta and Mor- ley (1973) Takashima <i>et al.</i> (1968) Fraefel and du Vigneaud (1970b)
Deamino-oxytocin ([Mpr ¹]-Oxytocin) [Mud ¹]-Oxytocin		

Table XXIII, *cont.*

Oxytocin, <i>cont.</i>	
[Mvl ¹]-Oxytocin	Fraefel and du Vigneaud (1970a)
Oxytocinyl-oxytocin	Takashima and du Vigneaud (1970)
Deamino-oxytocinyl-oxytocin	Takashima and du Vigneaud (1970)
[Ser ¹]-Oxytocin	Bayer <i>et al.</i> (1968); Bayer and Koenig (1969); Bayer (1970)
[Thr ¹]-Oxytocin	Manning and Sawyer (1970, 1972); Manning <i>et al.</i> (1970); Sawyer and Manning (1971)
[Gln ⁸]-Oxytocin	Baxter <i>et al.</i> (1969a)
[Phe ⁸]-Oxytocin	Baxter <i>et al.</i> (1969b)
[Gly ⁸]-Oxytocin	Havran <i>et al.</i> (1971b); Walter and Havran (1971)
Oxytocinoic acid	Beyerman <i>et al.</i> (1968a)
Oxytocinoic acid methylamide	Beyerman and Maassen van den Brink-Zimmermannová (1968)
N-Benzylloxycarbonyl-S,S'-dibenzylloxytocinoic acid hydrazide (hydroxamide)	Beyerman and Maassen van den Brink-Zimmermannová (1968)
N-Benzylloxycarbonyl-O-benzyl-S,S'-dibenzylloxytocinoic acid methyl ester	Beyerman <i>et al.</i> (1968b)
[Ile ¹]-Deamino-oxytocin	Takashima <i>et al.</i> (1970)
[Leu ¹]-Deamino-oxytocin	Takashima <i>et al.</i> (1970)
[Thr ¹]-Deamino-oxytocin	Manning and Sawyer (1972)
Deamino-oxytocinoic acid	Takashima and du Vigneaud (1970)
Deamino-oxytocinoic acid methylamide	Takashima <i>et al.</i> (1969)
Deamino-oxytocinoic acid dimethylamide	Takashima <i>et al.</i> (1969)
Acetone-oxytocinoic acid	Takashima and du Vigneaud (1970)
[Leu ⁸ , Thr ⁷]-Oxytocin	Manning and Sawyer (1972); Sawyer and Manning (1971)
[Pro ⁴ , Glu ⁸]-Oxytocin	Sawyer <i>et al.</i> (1969)
[Ser ⁴ , Glu ⁸]-Oxytocin (Glumitocin)	Manning <i>et al.</i> (1968a)
[Lys ⁴ , Ile ⁸]-Oxytocin	Sawyer and Manning (1971)
[Pro ⁴ , Ile ⁸]-Oxytocin	Sawyer <i>et al.</i> (1969)

[Thr ⁴ , Ile ⁵]-Oxytocin	Manning and Sawyer (1972); Sawyer and Manning (1971)								
[Pro ⁶ , Leu ⁸]-Oxytocin	Sawyer <i>et al.</i> (1969)								
Oxytocin-(5-9)-pentapeptide amide: Boc-Asn(Mbh)-Cys(Bzl)-Pro-Leu-Gly-NH ₂	Wissmann and Geiger (1970)								
Oxytocin-(6-9)-tetrapeptide amide: Z-Cys(Bzl)-Pro-Leu-Gly-NH ₂	Hruby <i>et al.</i> (1972)								
Oxytocin-(7-9)-tripeptide amide: Pro-Leu-Gly-NH ₂ Melanotropin-release inhibiting factor (see Celis <i>et al.</i> , 1971).	Bodanszky and Sheehan (1964); Mizoguchi <i>et al.</i> (1970); Beyerman <i>et al.</i> (1967b, 1968b)								
Oxytocin-(7-9)-tripeptide: Z-Pro-Leu-Gly-NH ₂	Garner <i>et al.</i> (1968)								
Oxytocin-(7-9)-tripeptide: Z-Pro-Leu-Gly-OR	Beyerman <i>et al.</i> (1968b, 1971a)								
R, methyl; ethyl; isopropyl									
Arginine-vasopressin									
Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Arg-Gly-NH ₂	1	2	3	4	5	6	7	8	9
Arginine-vasopressin	Meienhofer <i>et al.</i> (1970b, 1971); Lübke (1971)								
[Ile ³]-Arginine-vasopressin (Arginine-vasotocin)	Havran <i>et al.</i> (1971a)								
[Gly* ⁹]-Arginine-vasopressin	Walter and Havran (1971)								
Arginine-vasopressinoic acid	Wissmann and Geiger (1970); Meienhofer <i>et al.</i> (1971)								
Deamino-[Ile ³]-arginine-vasopressin	Havran <i>et al.</i> (1971a)								
[Ile ³]-Arginine vasopressinoic acid	Havran <i>et al.</i> (1971a)								
Lysine-vasopressin									
Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Lys-Gly-NH ₂	1	2	3	4	5	6	7	8	9
Lysine-vasopressin	Meienhofer and Sano (1968); Meienhofer and Trzeciak (1971)								
[Thr ⁴]-Lysine-vasopressin	Manning and Sawyer (1972)								
[Gly* ⁹]-Lysine-vasopressin	Havran <i>et al.</i> (1971b); Walter and Havran (1971)								
Lysine-vasopressin-(3-9)-heptapeptide amide	Meienhofer and Trzeciak (1970)								

Table XXIII, *cont.*

C. Other Peptide Hormones		
α -ACTH (<i>Adrenocorticotropin</i>)		[See Shepherd <i>et al.</i> (1956) and Li <i>et al.</i> (1958, 1961) for the structure of ACTH].
α -ACTH-(1-19)-nonapeptide: Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-Gly-Lys-Lys-Arg-Arg-Pro		Blake <i>et al.</i> (1972)
Alanyl- α -ACTH-(1-19)-nonapeptide		Blake <i>et al.</i> (1972)
Prolyl- α -ACTH-(1-19)-nonapeptide		Blake <i>et al.</i> (1972)
α -ACTH-(5-14)-decapeptide: Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-Gly		Yajima <i>et al.</i> (1970c)
α -ACTH-(11-19)-nonapeptide: Lys(Tos)-Pro-Val-Gly-Lys(Tos)-Lys(Tos)-Arg(Tos)-Arg(Tos)-Pro		Li and Hemmasi (1972)
<i>Calcitonin, human, M</i>		
Calcitonin-(8-10)-tripeptide: Boc-Met-Leu-Gly, (-NaH ₈)		[See Neher <i>et al.</i> (1968) and Sieber <i>et al.</i> (1968) for structure].
Calcitonin-(11-32)-docosapeptide: Boc-Thr-Tyr-Thr-Gln-Asn-Phe-Asn-Lys(Z)-Phe-His(Ztf)-Thr(Ztf)-Phe-Pro-Gln-Thr(Ztf)-Ala-Ile-Gly-Val-Gly-Ala-Pro-OMe		Kenner <i>et al.</i> (1971) Beyerman (1972)
Calcitonin-(24-30)-heptapeptide: Z-Gln-Thr-Ala-Ile-Gly-Val-Gly		Kenner <i>et al.</i> (1971)
<i>Gastrin</i>		
Gastrin-(14-17)-tetrapeptide amide: Trp-Met-Asp-Phe-NH ₂		[See Gregory <i>et al.</i> (1964) for structure]. Pietta and Marshall (1970); Flanigan and Marshall (1972); Shchukina <i>et al.</i> (1968)
<i>Glucagon</i> : His-Ser-Gln-Gly-Thr-Phe-Thr-Ser-Asp-Tyr-Ser- ⁵ ₁₀		[Structure of Bromer <i>et al.</i> (1956)]
Lys-Tyr-Leu-Asp-Ser-Arg-Arg-Ala-Gln-Asp-Phe-Val-Gln- ¹⁵ ₂₀		
Trp-Leu-Met-Asn-Thr ₂₅		

Glucagon-(16-29)-tetradecapeptide	Marshall (1968)
Glucagon-(18-29)-dodecapeptide	Southard <i>et al.</i> (1971c)
Glucagon-(24-29)-hexapeptide	Southard (1971)
[Gly ²⁸]-Glucagon-(18-22)-pentapeptide	Southard (1971)
<i>Growth hormone, human</i>	[Based on the originally proposed structure and residue numbers (Li <i>et al.</i> , 1966, 1969). For a revised structure, Li and Dixon (1971) and Niall (1971)].
<i>Growth hormone</i>	Li and Yamashiro (1970)
Growth hormone-(81-121)-hentetraconta peptide	Chillemi and Pecile (1971)
Growth hormone-(122-153)-dotriaconta peptide	Chillemi and Pecile (1971)
Growth hormone-(164-188)-pentacosapeptide	Bornstein <i>et al.</i> (1971)
Growth hormone-(1-21)-heneicosapeptide	Bornstein <i>et al.</i> (1971)
Growth hormone-(12-21)-decapeptide	Beyerman (1972)
Growth hormone-(17-23)-heptapeptide	(Revised structure) Yamashiro <i>et al.</i> (1972)
Growth hormone-(6-10)-pentapeptide	Beyerman (1972)
Growth hormone-(180-184)-pentapeptide	Marshall and Liener (1970)
Growth hormone-(131-134)-tetrapeptide	Dorman <i>et al.</i> (1972)
<i>β-Lipotropin, ovine</i>	[See Li <i>et al.</i> (1965) for structure]
<i>β-Lipotropin-(73-78)-hexapeptide</i>	Loffet <i>et al.</i> (1971)
<i>MSH, α-Melanotropin: Ac-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-</i>	
	1 5
Gly-Lys-Pro-Val-NH ₂	
10	
<i>α-Melanotropin</i>	
[Gln ⁵]- <i>α-Melanotropin</i>	Blake and Li (1971)
[Gln ⁵]- <i>α-Melanotropin</i> -(4-10)-heptapeptide	Blake <i>et al.</i> (1970)
<i>α-Melanotropin</i> -(6-10)-pentapeptide	Blake and Li (1968)
	Loffet (1967)

Table XXIII, *cont.*

<i>Parathyroid hormone, bovine</i>	[See Brewer and Ronan (1970) and Niall <i>et al.</i> (1970) for structure]
<i>Parathyroid hormone-(1-34)-tetratriacontapeptide</i>	Potts <i>et al.</i> (1971)
Ala-Val-Ser-Glu-Ile-Gln-Phe-Met-His-Asn-Glu-Gly-Lys-His-Leu- 1 5 10 15	
Ser-Ser-Met-Glu-Arg-Val-Glu-Trp-Leu-Arg-Lys-Lys-Leu-Gln-Asp- 20 25 30	
Val-His-Asn-Phe	
<i>Parathyroid Hormone-(19-25)-heptapeptide: Glu-Arg-Val-Glu-Trp-Leu-Arg</i>	Wang and Merrifield (1969b)
<i>Parathyroid Hormone-Related Peptide: Tyr-Lys-Glu-Leu-Val-Arg-Lys-Lys-Gly-Trp-His-Ile-Met-Glu-Ser-Phe-Ala-Val-Leu-Gln</i>	Yajima <i>et al.</i> (1970b)
<i>Secretin</i>	[See Mutt and Jorpes (1967) for structure]
Desamido-secretin	Bayer <i>et al.</i> (1970b)
Secretin-(1-6)-hexapeptide: His-Ser-Asp-Gly-Thr-Phe	Ondetti <i>et al.</i> (1968a)
Secretin-(22-27)-hexapeptide amide: Leu-Leu-Glu-Gly-Leu-Val-NH ₂	Bodanszky and Sheehan (1966); Bayer <i>et al.</i> (1971a)
<i>Thyrotropin releasing hormone, TRF, TRH: Glu-His-Pro-NH₂</i>	Flouret (1970); Baugh <i>et al.</i> (1970a); Beyerman <i>et al.</i> (1971b); Rivaillé and Milhaud (1971); Stewart <i>et al.</i> (1972)
α -Benzyl-[Glu ¹]-TRH	Flanigan and Marshall (1972)

Table XXIV—Kinins Prepared by Solid-Phase Peptide Synthesis

A. Angiotensins	
Angiotensinogen (renin substrate): Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu-Leu-Val-Tyr-Ser 1 5 10 14	
5-Isoleucine-angiotensin I: Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu 1 2 3 4 5 6 7 8 9 10	
5-Isoleucine-angiotensin II: Asp-Arg-Val-Tyr-Ile-His-Pro-Phe 1 2 3 4 5 6 7 8	
Angiotensinogen [Leu ^{*19}]-Angiotensinogen Ile ⁵ -Angiotensin I	Ryan (1970) Skeggs <i>et al.</i> (1969) Thampi and Huggins (1968); Thampi <i>et al.</i> (1968); Brunfeldt <i>et al.</i> (1969a); Oparil <i>et al.</i> (1972) Thampi <i>et al.</i> (1968) Oparil <i>et al.</i> (1972) Oparil <i>et al.</i> (1972)
[Leu ^{*19}]-Ile ⁵ -Angiotensin I [D-Leu ¹⁹]-Ile ⁵ -Angiotensin I [Des-Leu ¹⁹]-Ile ⁵ -Angiotensin I Ile ⁵ -Angiotensin II	Marshall and Merrifield (1965); Brunfeldt <i>et al.</i> (1969a); Bumpus <i>et al.</i> (1970); Chaturvedi <i>et al.</i> (1970); Fujii and Sakakibara (1970) Marshall and Merrifield (1965) Marshall and Merrifield (1965) Khosla <i>et al.</i> (1967b, 1968); Bumpus <i>et al.</i> (1970) Khosla <i>et al.</i> (1967a,b) Bumpus <i>et al.</i> (1970) Bumpus <i>et al.</i> (1970); Chaturvedi <i>et al.</i> (1970) Pisano (1969b) Bumpus <i>et al.</i> (1970) Bumpus <i>et al.</i> (1970); Chaturvedi <i>et al.</i> (1970)
[Asn ¹]-Ile ⁵ -Angiotensin II [β-Asp ¹]-Ile ⁵ -Angiotensin II [Ile ¹]-Ile ⁵ -Angiotensin II [Ala ¹]-Ile ⁵ -Angiotensin II [Pro ³]-Ile ⁵ -Angiotensin II [Tyr(Me) ¹]-Ile ⁵ -Angiotensin II [Ile ^{*6}]-Angiotensin II [Ala ⁹]-Ile ⁵ -Angiotensin II [Pip ⁷]-Ile ⁵ -Angiotensin II	

[Ala ⁸]-Ile ⁵ -Angiotensin II	Park <i>et al.</i> (1967)
[Apb ⁸]-Ile ⁵ -Angiotensin II	Bumpus <i>et al.</i> (1970); Chaturvedi <i>et al.</i> (1970)
[Apib ⁸]-Ile ⁵ -Angiotensin II	Bumpus <i>et al.</i> (1970); Chaturvedi <i>et al.</i> (1970)
[Pib ⁸]-Ile ⁵ -Angiotensin II	Semkin <i>et al.</i> (1967b)
[Tyr ⁸]-Ile ⁵ -Angiotensin II	Bumpus <i>et al.</i> (1970); Chaturvedi <i>et al.</i> (1970)
[Tyr(Me) ⁸]-Ile ⁵ -Angiotensin	Bumpus <i>et al.</i> (1970); Chaturvedi <i>et al.</i> (1970)
[Gly ⁷ , Gly ⁸]-Ile ⁵ -Angiotensin II (also N-acetyl deriv.)	Jorgensen <i>et al.</i> (1969, 1970c)
[Phe ⁴ , Tyr ⁸]-Ile ⁵ -Angiotensin II	Marshall <i>et al.</i> (1972)
[Gly ⁷ , Gly ⁸ , Gly ⁹]-Ile ⁵ -Angiotensin II	Jorgensen <i>et al.</i> (1970b)
Des-Asp ¹ -[R ⁷]-Ile ⁵ -Angiotensin II	Jorgensen <i>et al.</i> (1970a)
(R, <i>n</i> -C ₃ H ₇ CO, L-Abu, D-Abu, Acp, AvI, Gly, Ac-Gly)	
Succinyl-des-Asp ¹ -[Gly ⁷]-Ile ⁵ -Angiotensin II	Jorgensen <i>et al.</i> (1971a)
Aminooctanoyl-Val-Tyr-Ile-His-Pro-Phe	Jorgensen <i>et al.</i> (1970b)
Val ⁵ -Angiotensin I	Stewart <i>et al.</i> (1972)
Val ⁵ -Angiotensin II	Garner and Young (1969, 1971); Garner <i>et al.</i> (1971); Chaturvedi <i>et al.</i> (1970); Stewart <i>et al.</i> (1972)
[D-Val ⁷]-Angiotensin II	Marshall (1970)
[Asn ⁷]-Val ⁵ -Angiotensin II	Semkin <i>et al.</i> (1968)
[β-Asp ⁷]-Val ⁵ -Angiotensin II	Romanovskaya and Chipens (1971)
[Gly ⁷]-Val ⁵ -Angiotensin II	Pavars <i>et al.</i> (1971)
[Aib ⁷]-Val ⁵ -Angiotensin II	Marshall <i>et al.</i> (1972)
[Tyr(Me) ⁷]-Val ⁵ -Angiotensin II	Bumpus <i>et al.</i> (1970); Chaturvedi <i>et al.</i> (1970)
[MeAla ⁷]-Val ⁵ -Angiotensin II	Andreata and Scheraga (1971)
[Pro ⁷]-Val ⁵ -Angiotensin II	Marshall (1970)
[Tyr(Me) ⁸]-Val ⁵ -Angiotensin II	Bumpus <i>et al.</i> (1970); Chaturvedi <i>et al.</i> (1970)
[Asn ⁷ , HypIc ⁴]-Val ⁵ -Angiotensin II	Semkin <i>et al.</i> (1968)
[Asn ⁷ , Tyr(Me) ⁴]-Val ⁵ -Angiotensin II	Bumpus <i>et al.</i> (1970); Chaturvedi <i>et al.</i> (1970)
[Asn ⁷ , Pib ⁸]-Angiotensin II	Semkin <i>et al.</i> (1968)
[Phe ⁴ , Tyr ⁷]-Val ⁵ -Angiotensin II (specific inhibitor)	Marshall <i>et al.</i> (1970)
Des-(Asp ¹ , Arg ²)-Val ⁵ -Angiotensin II	Ovchinnikov <i>et al.</i> (1968b)

Table XXIV, *cont.*

A. Angiotensins, <i>cont.</i>	
5-Substituted angiotensin II analogs	
[Ala ⁵]-Angiotensin II	Khosla <i>et al.</i> (1968); Bumpus <i>et al.</i> (1970)
[Aib ⁵]-Angiotensin II	Marshall <i>et al.</i> (1972)
[Arg ⁵]-Angiotensin II	Paruszewski (1971)
[His ⁵]-Angiotensin II	Paruszewski (1971)
[Pab ⁵]-Angiotensin II	Paruszewski (1971)
[Pro ⁵]-Angiotensin II	Paruszewski (1971)
[Ser ⁵]-Angiotensin II	Paruszewski (1971)
[Tyr ⁵]-Angiotensin II	Paruszewski (1971)
[D-Val ⁵]-Angiotensin II	Marshall (1970)
[d-Val ⁵]-Angiotensin II	Marshall (1970)
[Asn ¹ , Ala ⁷]-Angiotensin II	Jorgensen and Weinkam (1973); Jorgensen <i>et al.</i> (1971b)
[Asn ¹ , alle ⁷]-Angiotensin II	Jorgensen and Weinkam (1973); Jorgensen <i>et al.</i> (1971b)
[Asn ¹ , Abu ⁷]-Angiotensin II	Jorgensen and Weinkam (1973); Jorgensen <i>et al.</i> (1971b)
[Asn ¹ , Aev ⁷]-Angiotensin II	Jorgensen and Weinkam (1973); Jorgensen <i>et al.</i> (1971b)
[Asn ¹ , Aib ⁷]-Angiotensin II	Jorgensen and Weinkam (1973); Jorgensen <i>et al.</i> (1971c)
[Asn ¹ , Chg ⁷]-Angiotensin II	Jorgensen and Weinkam (1973); Jorgensen <i>et al.</i> (1971b)
[Asn ¹ , Cle ⁷]-Angiotensin II	Jorgensen and Weinkam (1973); Jorgensen <i>et al.</i> (1971c)
[Asn ¹ , Cpg ⁷]-Angiotensin II	Jorgensen and Weinkam (1973); Jorgensen <i>et al.</i> (1971b)
[Asn ¹ , Hyv ⁷]-Angiotensin II	Semkin <i>et al.</i> (1968)
[Asn ¹ , Nle ⁷]-Angiotensin II	Jorgensen and Weinkam (1973); Jorgensen <i>et al.</i> (1971b)
[Asn ¹ , Pro ⁷]-Angiotensin II	Jorgensen and Weinkam (1973); Jorgensen <i>et al.</i> (1971c)
[Asn ¹ , D-Pro ⁷]-Angiotensin II	Jorgensen and Weinkam (1973); Jorgensen <i>et al.</i> (1971c)
[Asn ¹ , Sar ⁷]-Angiotensin II	Jorgensen and Weinkam (1973); Jorgensen <i>et al.</i> (1971c)

B. Bradykinin, Eledoisin, Physalaemin

Bradykinin: H-Arg-Pro-Gly-Phe-Ser-Pro-Phe-Arg-OH

1 2 3 4 5 6 7 8 9

Bradykinin	Merrifield (1964a,b, 1965b, 1966, 1967); Merrifield and Stewart (1965); Fridkin <i>et al.</i> (1966b, 1968); Najjar and Merrifield (1966); Lenard and Robinson (1967); Patchornik <i>et al.</i> (1967); Brunfeldt <i>et al.</i> (1968, 1969a); Melrose <i>et al.</i> (1970); Garner <i>et al.</i> (1971); Schafer <i>et al.</i> (1971); Sipos and Gaston (1971)
Acetyl-bradykinin	Stewart and Woolley (1965b, 1966)
Angiotensinyl-bradykinin	Merrifield (1966, 1967)
Bradykininyl-bradykinin	Najjar and Merrifield (1966)
<Glu-Thr-Asn-Lys-Lys-Leu-Arg-Gly-Bradykinin	Stewart (1968)
[Ala ¹]-Bradykinin	Melrose <i>et al.</i> (1970)
[D-Pro ²]-Bradykinin	Stewart and Woolley (1966)
[D-Pro ³]-Bradykinin	Stewart and Woolley (1966)
[Cha ⁵]-Bradykinin	Elliott <i>et al.</i> (1973)
[Thi ⁶]-Bradykinin	Dunn and Stewart (1971)
[Lys(Tos) ⁹]-Bradykinin	Stewart and Woolley (1966)
[Phe ⁸]-Bradykinin	Stewart and Woolley (1966)
[Thr ⁸]-Bradykinin	Stewart and Woolley (1966)
[D-Pro ⁷]-Bradykinin	Stewart and Woolley (1966)
[Cha ⁸]-Bradykinin	Elliott <i>et al.</i> (1973)
[Thi ⁸]-Bradykinin	Dunn and Stewart (1971)
Endo-Phe ⁸ -Bradykinin	Stewart and Woolley (1966)
[D-Arg ⁷]-Bradykinin	Stewart and Woolley (1966)
Bradykininyl-arginine	Stewart and Woolley (1965b, 1966)
Bradykininyl-glycine	Stewart and Woolley (1965b, 1966)
Acetyl-[D-Pro ⁷]-Bradykinin	Stewart and Woolley (1966)

Table XXIV, *cont.*

B. Bradykinin, Eleodoisin, Physalaemin, <i>cont.</i>	
[Pro ²² , Pro ²³]-Bradykinin	Pisano (1969a)
[Cha ⁵ , Cha ⁹]-Bradykinin	Elliott <i>et al.</i> (1973)
[Thr ⁵ , Thr ⁹]-Bradykinin	Dunn and Stewart (1971)
[Tyr(Me) ⁵ , Tyr(Me) ⁹]-Bradykinin (also N-acetyl derivative)	Stewart and Woolley (1966)
[Thr ⁶ , Leu ⁹]-Bradykinin	Stewart and Woolley (1966)
[Gly ⁶ , Tyr(Me) ⁹]-Bradykinin (also N-acetyl derivative)	Stewart and Woolley (1966)
Glycyl-[Gly ⁶ , Tyr(Me) ⁹]-Bradykinin	Stewart and Woolley (1966)
[D-Pro ² , D-Pro ³ , D-Pro ⁷]-Bradykinin	Stewart and Woolley (1966)
[Leu ⁶ , Gly ⁶ , Leu ⁹]-Bradykinin	Stewart and Woolley (1966)
[Leu ⁵ , Thr ⁶ , Leu ⁹]-Bradykinin	Stewart and Woolley (1966)
[Tyr(Me) ⁵ , Asn ⁶ , Tyr(Me) ⁹]-Bradykinin	Stewart and Woolley (1966)
[Tyr(Me) ⁵ , Gly ⁶ , Tyr(Me) ⁹]-Bradykinin	Stewart and Woolley (1966)
[Tyr(Me) ⁵ , Thr ⁶ , Tyr(Me) ⁹]-Bradykinin	Stewart <i>et al.</i> (1967)
O ¹⁰ -(Arg-Pro-Pro-Gly)-[Tyr(Me) ⁵ , Tyr(Me) ⁹]-Bradykinin	Stewart <i>et al.</i> (1967)
[Tyr(Me) ⁵ , Tyr(Me) ⁹]-Bradykininyl-glycine	Stewart and Woolley (1966)
[Tyr(Me) ⁵ , Tyr(Me) ⁹]-Bradykininyl-phenylalanine	Stewart and Woolley (1966)
Des-Arg ² -[Tyr(Me) ⁵ , Thr ⁶ , Leu ⁹]-Bradykinin	Stewart and Woolley (1966)
[D-Arg ² , D-Phe ⁵ , D-Ser ⁶ , D-Phe ⁶ , D-Arg ⁹]-Bradykinin	Stewart and Woolley (1966)
Des-Arg ² -[D-Pro ² , D-Pro ³ , Tyr(Me) ⁵ , D-Pro ⁷ , Tyr(Me) ⁹]-Bradykinin	Stewart and Woolley (1963a)
All-D-Bradykinin	Stewart and Woolley (1966)
All -D-Retrobradykinin	Brunfeldt <i>et al.</i> (1969a)
Kallidin (Lysyl-bradykinin)	Pisano (1969b)
[¹⁴ C-Pro ³ , ¹⁴ C-Pro ⁷]-Kallidin	Merrifield (1964c)
Methionyllysyl-bradykinin	
Eleodoisin: <Glu-Pro-Ser-Lys-Asp-Ala-Phe-Ile-Gly-Leu-Met-NH ₂	
	1 2 3 4 5 6 7 8 9 10 11

Eleodoisin-(6-11)-hexapeptide [Lys ⁶]-Eleodoisin-(5-11)-heptapeptide [Gly ⁶ , Val ⁸]-Eleodoisin-(5-11)-heptapeptide	Norris <i>et al.</i> (1971) Halstrøm <i>et al.</i> (1969) Shchukina and Sklyarov (1966)
Physalaemin: Glu-Ala-Asp-Pro-Asn-Lys-Phe-Tyr-Gly-Leu-Met-NH ₂ 1 2 3 4 5 6 7 8 9 10 11	
Bis-(<i>tert</i> -Amyloxycarbonyl)-physalaemin-(6-11) <i>tert</i> -Amyloxycarbonyl-[Phe ⁶ , Tyr(Me) ⁸]-physalaemin-(6-11) <i>tert</i> -Amyloxycarbonyl-[Phe ^{6,8}]-physalaemin-(6-11)	Inukai <i>et al.</i> (1968) Inukai <i>et al.</i> (1968) Inukai <i>et al.</i> (1968)
Substance P: Arg-Pro-Lys-Pro-Gln-Phe-Phe-Gly-Leu-Met-NH ₂ [Structure, see Chang <i>et al.</i> (1971)]	
Substance P	Tregear <i>et al.</i> (1971)

Table XXV—Other Biologically Active Peptides and Proteins Prepared by Solid-Phase Synthesis

A. Antibiotics, toxins, and related peptides	
<i>Antamanide</i> : [Val-Pro-Ala-Phe-Phe-Pro-Phe-Phe] 1 5 10	Wieland <i>et al.</i> (1969a); Birr and Lochinger (1971); Birr <i>et al.</i> (1973); Wieland <i>et al.</i> (1971b)
[Abu ¹]-Antamanide	Wieland <i>et al.</i> (1971c)
[Abu ¹]-Antamanide	Wieland <i>et al.</i> (1971c)
[Abu ¹ , Abu ⁴]-Antamanide	Wieland <i>et al.</i> (1971c)
[Phe ⁴ , Val ⁹]-Antamanide	Wieland <i>et al.</i> (1971a)
Linear decapeptide: Phe-Pro-Pro-Phe-Phe-Val-Pro-Pro-Ala-Phe 6 10 1 5	Wieland <i>et al.</i> (1970)
<i>Bothrops Jararaca Venom</i> Pentapeptide (BJV): Glu-Lys-Trp-Ala-Pro	Greene <i>et al.</i> (1970)
[Pro ¹]-BJV	Freer and Stewart (1972)
2-Acetyl-BJV	Freer and Stewart (1972)
BJV-amide	Freer and Stewart (1972)
<Glu-[Pro ¹]-BJV	Freer and Stewart (1972)
Pbu-[Pro ¹]-BJV	Freer and Stewart (1972)
BJV-Phe-Arg	Freer and Stewart (1972)
BJV-Ile-Pro-Pro	Freer and Stewart (1972)
<Glu-Trp-Pro-Arg-Pro	Freer and Stewart (1972)
<Glu-Lys-Trp-Pro-Arg-Pro	Freer and Stewart (1972)
<Glu-Pro-Pro-Gly-Trp-Ser	Freer and Stewart (1972)
<Glu-Pro-Gly-Trp-Ser-Pro	Freer and Stewart (1972)
Cobrotoxin-(47-52)-hexapeptide: Lys-Asn-Gly-Ile-Gly-Ile (<i>Naja naja atra</i>)	Noda <i>et al.</i> (1970b)

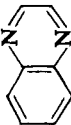
<i>Gramicidin A</i> : For-Val-Gly-Ala-D-Leu-Ala-D-Val-Val-D-Val- 1 5 Trp-D-Leu-Trp-D-Leu-Trp-D-Leu-Trp- 10 15 NHCH ₂ CH ₂ OH	Gross (1971)
<i>Gramicidin B</i> : ([Phe ¹¹]-Gramicidin A)	Gross (1971)
<i>Gramicidin S</i> : [Val-Orn-Leu-D-Phe-Pro-Val-Orn-Leu-D-Phe-Pro- 1 5 10	Klostermeyer (1968); Losse and Neubert (1970b); Ohno <i>et al.</i> (1971)
[Gly ⁴ , Gly ¹⁰]-Gramicidin S	Kiryushkin <i>et al.</i> (1967); Klostermeyer <i>et al.</i> (1967); Halstrøm and Klostermeyer (1968); Ovchinnikov <i>et al.</i> (1968b); Sklyarov and Sbashkova (1969)
Z-Val-Orn(Tfa)-Leu-D-Phe-Pro-OMe (derivatives)	Fridkin <i>et al.</i> (1971)
<i>Melittin</i> (bee venom)	[See Habermann and Jentsch (1967) for structure]
Melittin-(1-17)-heptadecapeptide: Gly-Ile-Gly-Ala-Val- Leu-Lys-Val-Leu-Thr-Thr-Gly-Leu-Pro-Ala-Leu-Ile	Dorman and Markley (1971)
Melittin-(1-9)-nonapeptide: Gly-Ile-Gly-Ala-Val-Leu-Lys- Val-Leu	Miura <i>et al.</i> (1968)
Penicillin precursor: Aad Cys-[¹⁴ C]-Val	Bauer (1970)
<i>Quinomycin, triostin</i>	[See Keller-Schierlein <i>et al.</i> (1959); Otsuka and Shoji (1967) for structure]
Model:  CO-Ser-Ala-Cys(Bzl)-Val	Olsen (1970)
<i>Polymyxin</i> (partial sequences of lysine-containing analogs)	[Review on Polymyxin structures, see Vogler and Studer (1966)]
<i>Polymyxin B</i> : Pel-Lys(Boc)-Lys(Z)-D-Phe-Leu-Lys(Z)-Lys(Z)-Thr	Morozova and Zevail (1970a)

Table XXV, *cont.*

A. Antibiotics, toxins, and related peptides, <i>cont.</i>	
<i>Polymyxin D</i> : Boc-Thr-Lys(Z)-Lys(Z)-Thr- $\square$ Pel-Lys-Lys(Z)-D-Leu	Zevail and Morozova (1971)
<i>Polymyxin E</i> : Boc-Leu-Lys(Z)-Lys(Z)-Thr- $\square$ Pel-Lys-Lys(Z)-D-Leu	Zevail and Morozova (1970)
<i>Polymyxin M</i> : Boc-Lys(Z)-Thr- $\square$ Pel-Lys-Lys(Z)-Thr-Lys(Z)-D-Leu	Morozova and Zevail (1970b); Morozova <i>et al.</i> (1970)
Valinomycin: <i>cyclo</i> (-L-Val-Hyv-D-Val-Lac-L-Val-Hyv-D-Val-Lac-L-Val-Hyv-D-Val-Lac-) Lac-L-Val-Hyv-D-Val-Lac-)	Gisin <i>et al.</i> (1969); Gisin and Merrifield (1970); Losse and Klen- gel (1971)
B. Enzymes	
<i>Acyl carrier protein, E. coli</i>	[Structure, See Vanaman <i>et al.</i> (1968)]
<i>Acyl carrier protein</i> -(1-74)	Hancock <i>et al.</i> (1971); Marshall <i>et al.</i> (1973)
<i>Acyl carrier protein</i> -(33-37)-pentapeptide: Gly-Ala-Asp-Ser-Leu	Miura and Seto (1969)
<i>Chymotrypsin</i>	[Structure, see Brown and Hartley (1966); Hartley and Kauffman (1966); Meloun <i>et al.</i> (1966)]
<i>Chymotrypsin</i> -(193-201)-nonapeptide: Gly-Asp-Ser-Gly-Gly- Pro-Leu-Val-Cys(Bzl)	Mizoguchi and Woolley (1967)
<i>Cytochrome c</i> , horse heart [Phe ⁴⁹]-Cytochrome c, equine Cytochrome c-(56-104)-nonatetracontapeptide, equine Cytochrome c-(1-29)-nonacosapeptide, equine	[Structure, see Margoliash <i>et al.</i> (1961)] Sano and Kurihara (1969) Sano <i>et al.</i> (1968) Sano <i>et al.</i> (1968)

Cytochrome c-(10-19)-decapeptide, equine: Val-Gln-Lys-Cys-Ala-Glu-Cys-His-Thr-Val	Sano <i>et al.</i> (1968)
[Arg ²⁷]-Cytochrome c-(13-22)-decapeptide, equine: Lys-Cys-Ala-Gln-Cys-His-Thr-Val-Glu-Arg	Robinson and Kamen (1963)
Cytochrome c-(14-20)-heptapeptide, equine: Cys-Ala-Gln-Cys-Thr-His-Val	Sano <i>et al.</i> (1968)
Cytochrome b ₅ -tetradecapeptide	Lvova <i>et al.</i> (1971)
Cytochrome b ₅ -(26-34)-nonapeptide: Lys(Tos)-Phe-Leu-Glu-Glu-His(Bzl)-Pro-Gly-Gly	Kozhukhovskaya <i>et al.</i> (1970)
Cytochrome c, yeast	[Structure, see Yaoi <i>et al.</i> (1966)]
Cytochrome c-(14-22)-nonapeptide	Alarkon <i>et al.</i> (1971)
Cytochrome c-(11-17)-heptapeptide	Alarkon <i>et al.</i> (1971)
Cytochrome related peptides: Cys-Ala-Val-Cys-His-Ala-Val-Ala-Lys-Ala-Ala-Ala-Ala-Ala	Robinson and Kamen (1968)
Cytochrome related peptides: Lys-Cys-Ala-Val-Cys-Arg	Robinson and Kamen (1968)
<i>Ferredoxin, Clostridium pasteurianum</i>	[Structure, see Tanaka <i>et al.</i> (1966)]
Apoferredoxin	Bayer <i>et al.</i> (1968a,b)
Ferredoxin (19-31)-tridecapeptide: Pro-Val-Asn-Ala-Ile-Ser-Gln-Glu-Asp-Ser-Ile-Phe-Val	Kelly and Levy (1971)
Ferredoxin (Clostr. butyricum)-(48-55)-octapeptide: Pro-Val-Gly-Ala-Pro-Asn-Gln-Glu	B. Mitchell <i>et al.</i> (1970)
Ferredoxin-(48-55)-octapeptide: Pro-Val-Gly-Ala-Pro-Val-Gln-Glu	Mitchell and Levy (1970); B. Mitchell <i>et al.</i> (1970)
Ferredoxin (1-7)-heptapeptide: Ala-Tyr-Lys-Ile-Ala-Asp-Ser	Kelly and Levy (1971)
Ferredoxin-(31-36)-hexapeptide: Val-Ile-Asp-Ala-Asp-Thr	B. Mitchell <i>et al.</i> (1970)
<i>Lysozyme</i> , egg-white, hen	[Structure, see Hill (1968)]
<i>Lysozyme</i>	Barstow <i>et al.</i> (1971)
<i>Lysozyme</i> -(74-85)-decapeptide: Asn-Leu-Cys(CM)-Asn-Ile-Pro-Cys(CM)-Ser-Ala-Leu	Thompson and Levy (1970)

Table XXV, *cont.*

B. Enzymes, <i>cont.</i>	
Lyzozyme-(64-71)-octapeptide: Cys(Bzl)-Asn-Asp-Gly-Arg(NO ₂)-Thr-Pro-Gly	Jollès and Jollès (1968)
Lyzozyme-(124-129)-hexapeptide: Ile-Arg-Gly-Cys(CM)-Arg-Leu	Hruby <i>et al.</i> (1972)
<i>Nuclease, Staphylococcus aureus</i>	[Structure, see Taniuchi <i>et al.</i> (1967, 1968); Cusumano <i>et al.</i> (1968)]
Nuclease-(6-47): Lys-Leu-His-Lys-Glu-Pro-Ala-Thr-Leu-Ile- Lys-Ala-Ile-Asp-Gly-Asp-Thr-Val-Lys-Leu- Met-Tyr-Lys-Gly-Gln-Pro-Met-Thr-Phe-Arg- Leu-Leu-Leu-Val-Asp-Thr-Pro-Glu-Thr-Lys- His-Pro	10 15 20 25 30 35 40 45
[Asn ²¹]-Nuclease-(6-47)	Chaiken and Anfinsen (1971)
[Glu ²¹]-Nuclease-(6-47)	Chaiken and Anfinsen (1971)
[Ile ²¹]-Nuclease-(6-47)	Ontjes and Anfinsen (1969b)
[Ile ²³]-Nuclease-(6-47)	Ontjes and Anfinsen (1969b)
[Cit ²⁶]-Nuclease-(6-47)	Chaiken and Anfinsen (1971)
[Lys ²⁶]-Nuclease-(6-47)	Chaiken and Anfinsen (1971)
[Asn ⁴⁰]-Nuclease-(6-47)	Chaiken and Anfinsen (1971)
[Glu ⁴⁰]-Nuclease-(6-47)	Chaiken and Anfinsen (1971)
[Asp ⁴⁹]-Nuclease-(6-47)	Chaiken and Anfinsen (1971)
[Gln ⁴³]-Nuclease-(6-47)	Chaiken and Anfinsen (1971)
[Gly ⁴⁵]-Nuclease-(6-47)	Chaiken and Anfinsen (1970a)
Nuclease-(9-47)-nonatriacontapeptide	Ontjes and Anfinsen (1969b); Chaiken and Anfinsen (1970b)

[Gly ⁴⁶]-Nuclease-(9-47)-nonatriacontapeptide	Chaiken and Anfinsen (1970b)
Nuclease-(10-47), -(11-47), -(12-47), -(16-47)-dotriacontapeptide	Chaiken and Anfinsen (1970b)
Nuclease-(18-47)-triacontapeptide	Ontjes and Anfinsen (1969b); Chaiken and Anfinsen (1970b)
Nuclease-(33-47)-pentadecapeptide	Ontjes and Anfinsen (1969b)
Nuclease-(108-117)-decapeptide: Leu-Ala-Lys-Val-Ala-Tyr-Val-Tyr-Lys-Pro	Anfinsen <i>et al.</i> (1967)
Nuclease-(132-139)-octapeptide: Glu-Ala-Gln-Ala-Lys-Leu-Asn-Ile	Anfinsen <i>et al.</i> (1967); Ohno <i>et al.</i> (1969)
Nuclease-(108-113)-Gly: Leu-Ala-Lys-Val-Ala-Tyr-Gly	Anfinsen (1968)
Nuclease-(89-94)-hexapeptide: Leu-Ala-Tyr-Ile-Tyr-Ala	Anfinsen (1968)
Nuclease-(52-56)-pentapeptide: Glu-Lys-Tyr-Gly-Pro	Anfinsen (1968)
<i>Ribonuclease T₁, Aspergillus oryzae</i>	[Structure, see Takahashi (1965)]
<i>Ribonuclease T₁</i>	Izumiya <i>et al.</i> (1971)
<i>Ribonuclease A, bovine</i>	[Structure, see Hirs <i>et al.</i> (1960); Smyth <i>et al.</i> (1963)]
<i>Ribonuclease A</i>	Gutte and Merrifield (1969, 1971)
S-Protein [Ribonuclease A-(21-124)]	Gutte and Merrifield (1970)
Des(21-25)-S-Protein [Ribonuclease A-(26-124)]	Gutte and Merrifield (1970)
Ribonuclease A-(1-20), S-Peptide	Gutte and Merrifield (1971)
Ribonuclease A-(1-15)-pentadecapeptide	Kato and Anfinsen (1969)
[¹⁴ C-Phe ⁸]-Ribonuclease A-(1-15)-pentadecapeptide	Freedman <i>et al.</i> (1971)
Ribonuclease A-(111-124)-tetradecapeptide	Lin <i>et al.</i> (1970)
[Leu ¹³]-Ribonuclease A-(1-13)-tridecapeptide	Visser <i>et al.</i> (1970a)
[Cit ¹⁰ , Leu ¹³]-Ribonuclease A-(1-13)-tridecapeptide	Visser and Kerling (1970)
[Har ⁶ , Leu ¹²]-Ribonuclease A-(1-13)-tridecapeptide	Visser <i>et al.</i> (1970b)
Ribonuclease A-(1-8)-octapeptide	Visser <i>et al.</i> (1968)

Table XXV, cont.

B. Enzymes, cont.	
<i>Proteinase, Streptococcus</i> (Peptide around the sulfhydryl group)	[Structure, see Liu <i>et al.</i> (1965)]
Heptacosapeptide: Val-Lys-Pro-Gly-Glu-Gln-Ser-Phe-Val-Gly-Gln-Ala-Ala-Thr-Gly-His-Cys-Val-Ala-Thr-Ala-Thr-Ala-Gln-Ile-Met-Lys	Schaich and Schneider (1973)
Hexapeptide: Z-Glu(OBu')-Gln-Ser(Bzl)-Phe-Val-Gly-OMe	Schaich and Schneider (1971)
Hexapeptide: Boc-Thr(Bzl)-Ala-Gln-Ile-Met-Lys(Z)-NH ₂	Schaich and Schneider (1971)
Pentapeptide: Boc-Gln-Ala-Ala-Thr(Bzl)-Ala-N ₂ H ₅	Schaich and Schneider (1971)
Tetrapeptide: Boc-Val-Lys(Z)-Pro-Gly-N ₂ H ₅	Schaich and Schneider (1971)
<i>Trypsin Inhibitor</i> , Basic, bovine	[Structure, see Kassell and Laskowski (1965)]
<i>Trypsin Inhibitor</i>	Izumiya <i>et al.</i> (1972); Noda <i>et al.</i> (1971)
C. Other Biologically Active Peptides	
<i>Bacterial Cell Wall</i> : B ₁ anthrax: deca-γ-D-Glu	Nitecki and Goodman (1970)
<i>α-Casein</i>	[General Information, see Schormüller <i>et al.</i> (1966)]
Partial Sequences: Val-Ser-Ile-Glu-Glu-Ser-Ser-Pro-Ser-Val-Glu-Glu-Glu-Asp-Ser-Ile-Ala	Polzhofer and Ney (1969, 1970a)
Lys-Asp-Glu-Glu-Glu-Val-Glu-Ser-Phe-Ser-Gly-Pro-Asp-Ala-Pro-Leu-Pro-Ala-Gly	Polzhofer and Ney (1969)
Val-Asp-Glu-Glu-Glu-D,L-Ser-Ile-Ala-Met-Glu-Lys	Ney and Polzhofer (1968)
Val-Ser-Glu-Glu-Asp-Ile-Ala-Glu-Met-Lys	Polzhofer and Ney (1969)
Ser-Pro-Pro-Gly	Polzhofer and Ney (1969)
Ala-Ser-Phe	Polzhofer and Ney (1969)

Ala-Thr-Phe κ-Casein: Lys-His-Pro-Pro-His-Leu-Ser-Phe-Met-Ala-Ile-Pro-Pro-Lys-Lys	Polzhofer and Ney (1969) Polzhofer (1971)
<i>Encephalitogenic A₁ Protein from Myelin, bovine</i> Partial Sequences: Ser-Arg-Phe-Trp-Gly-Ala-Glu-Gly-Gln-Lys- 1 5 10 Analog: [Val ¹], [Ala ⁴], [Phe ⁵], [Val ⁶], [Ile ⁸], [Ile ¹⁰], [Arg ¹¹], [Ile ¹¹]-analogs Ser-Arg-Phe-Gly-Ser-Trp-Gly-Ala-Glu-Gly-Gln-Ser-Pro-Phe-Gly-Lys Encephalytogenic basic myelin protein-(66-75)-decapeptide: Thr-Thr-His-Tyr-Gly-Ser-Leu-Pro-Gln-Lys [Gly ⁷⁵]-Encephalytogenic basic myelin protein-(64-76)-tridecapeptide: Ala-Arg-Thr-Thr-His-Tyr-Gly-Ser-Leu-Pro-Gln-Lys-Gly	[Structure, see Eylar <i>et al.</i> (1971)] Eylar <i>et al.</i> (1970); Westall <i>et al.</i> (1971) Westall <i>et al.</i> (1971) Westall and Robinson (1970) Shapira <i>et al.</i> (1971) Shapira <i>et al.</i> (1971)
<i>Fibrinopeptide A, human:</i> Ala-Asp-Ser-Gly-Glu-Gly-Asp-Phe-Leu-Ala-Glu-Gly-Gly-Gly-Val-Arg	Johnson and May (1969)
<i>Folic Acid Conjugates, Vitamin B₆</i> Pteroyloloigo-γ-glutamates	[General Information, see Pfiffner <i>et al.</i> (1946)] Krumdieck and Baugh (1969)

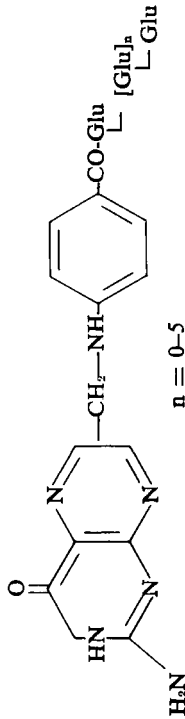


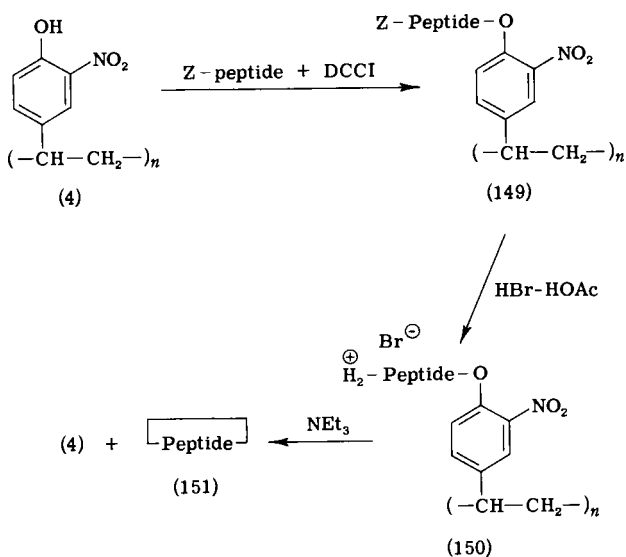
Table XXV, *cont.*

C. Other Biologically Active Peptides, <i>cont.</i>		
Oligo- γ -glutamates $n = 1,3,5$		Meienhofer <i>et al.</i> (1970a)
Pteroyl-Glu └─ Glu └─ R	R: Asp, Gly, Leu, Phe, Pro, Ser, Thr, Val, Leu-Leu	Baugh <i>et al.</i> (1970b)
Glutathione: Z-Glu-OBzl └─ Cys(Z)-Gly-OBzl		Fridkin <i>et al.</i> (1966a); Patchornik <i>et al.</i> (1967)
Z-Glu └─ Cys(Bzl)-Gly		Inukai <i>et al.</i> (1968)
Hapten: (D-Ala-Ala) ₅ -Lys(Dnp)		Richards <i>et al.</i> (1966, 1967)
Hemoglobin, human		[Structure, see Braunitzer <i>et al.</i> (1961, 1964)]
α -Chain-(1-7)-heptapeptide: Val-Leu-Ser-Pro-Ala-Asp-Lys		Tritsch and Grahl-Nielsen (1969)
β -Chain-(124-146)-tricosapeptide: Pro-Pro-Val-Gln-Ala-Ala-Tyr-Gln-Lys-Val-Val-Ala-Gly-Val-Ala-Asn-Ala-Leu-Ala-His-Lys-Thr-His		Chillemi and Merrifield (1969); Chillemi (1971)
Myoglobin, sperm whale		[Structure, see Edmundson (1965)]
Myoglobin-(147-153)-heptapeptide: Lys-Glu-Leu-Gly-Tyr-Gln-Gly		Givas <i>et al.</i> (1968)
Peptidoglycan peptides, <i>Streptococci</i> (related)		
Gly-Gly-Ala-D-Ala		Schleifer and Krause (1971)
Gly-Gly-Ala-D-Ala-D-Ala		Schleifer and Krause (1971)

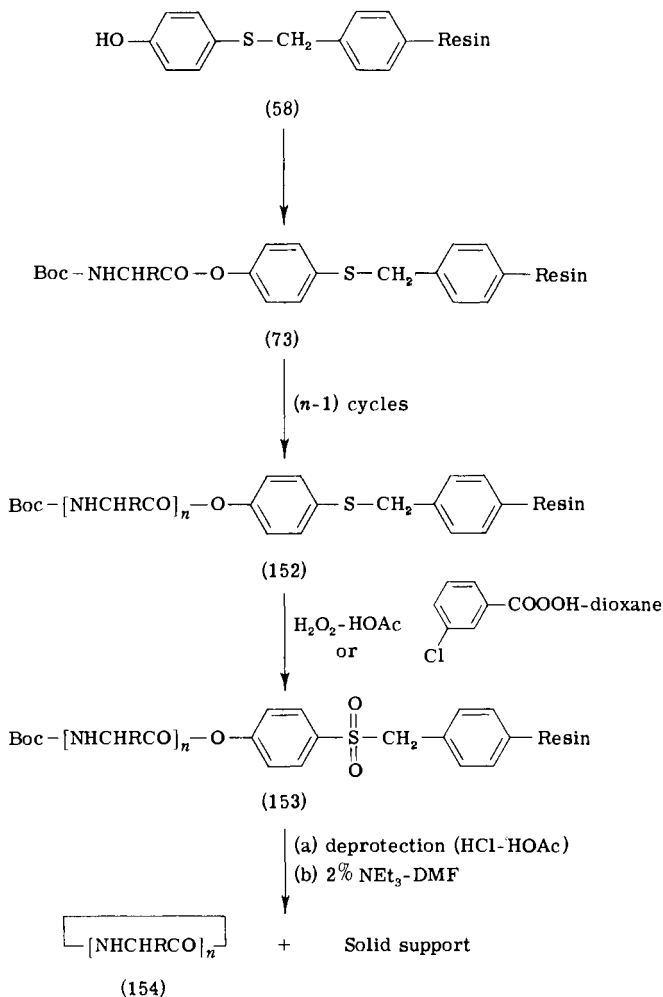
Gly-Gly-Lys-D-Ala-D-Ala	Schleifer and Krause (1971)
Gly-D-Glu	Schleifer and Krause (1971)
└ Ala-D-Ala-D-Ala	
Ala-D-Glu	Schleifer and Krause (1971)
└ Lys-D-Ala-D-Ala	
Gly-D-Glu	Schleifer and Krause (1971)
└ Lys-D-Ala-D-Ala	
Ala-D-Glu	Schleifer and Krause (1971)
└ Lys-D-Ala	
<i>Tobacco Mosaic Virus Protein</i> (Vulgare)	[Structure, see Funatsu <i>et al.</i> (1964); Anderer <i>et al.</i> (1965)]
TMV-Protein-(93-112)-eicosapeptide: Ile-Ile-Glu-Val-Glu-Asn-Gln-Ala-Asn-Pro-Thr-Thr-Ala-Glu-Thr-Leu-Asp-Ala-Thr-Arg	Young <i>et al.</i> (1972)
TMV-Protein-(103-112)-decapeptide: Thr-Thr-Ala-Glu-Thr-Leu-Asp-Ala-Thr-Arg	Young <i>et al.</i> (1967, 1968b); Spittler <i>et al.</i> (1970)
TMV-Protein-(42-46)-pentapeptide: Thr-Val-Val-Gln-Arg	Young <i>et al.</i> (1968a)
Analog: Thr-Val-Gln-Val-Arg	Young <i>et al.</i> (1968a)
TMV-Protein-(81-85)-pentapeptide: Z-Thr-Ala-Leu-Leu-Gly-N ₃ H ₄	Pettit and Jones (1971)
TMV-Protein-(98-102)-pentapeptide: Asp(ONb)-Glu(ONb)-Ala-Asp(ONb)-Pro	Brunfeldt and Halstrøm (1970)
TMV-Protein-(108-112)-pentapeptide: Leu-Asp-Ala-Thr-Arg	Young <i>et al.</i> (1972)
Analog: (Lys)-Leu-Asp-Ala-Thr-Arg	Spittler <i>et al.</i> (1970)
Over 20 Pentapeptide Analogs	Benjamini <i>et al.</i> (1968a,b)
But-Ala-Thr-Arg	Spittler <i>et al.</i> (1970)
Dec-Ala-Thr-Arg	Spittler <i>et al.</i> (1970)
Hex-Ala-Thr-Arg	Spittler <i>et al.</i> (1970)
Oct-Ala-Thr-Arg	Spittler <i>et al.</i> (1970)

In cases where the labeled amino acid residue is introduced at or near the C-terminal, the advantage of working in one vessel throughout the entire synthesis until the product is cleaved from the solid support is all the more apparent. Radioactively labeled peptides prepared by the solid-phase method are listed in Table XXVI.

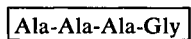
Peptide cyclizations are generally carried out in the lowest concentrations feasible to minimize intermolecular reaction. The solid-phase method provides an ideal system for cyclization since the dilution is extremely high. In fact, in the ideal situation all molecules are isolated from each other with no means of interaction. Fridkin *et al.* (1965a) took advantage of this system to prepare cyclic peptides. N-Protected peptides were attached (149) to copoly-4-hydroxy-3-nitrostyrene divinylbenzene (4). Removal of the N-protecting group [to give (150)] and base treatment produced cyclic peptides (151).



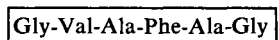
Flanigan and Marshall (1972) used the "dual function" methylthiophenyl ester resin (58) to synthesize resin-bound peptides (152) which were subsequently cyclized (154) and removed from the support after oxidative conversion to the activated methylsulfonyl ester type grouping (153) and N-deprotection.



The cyclic tetrapeptide



and the cyclic hexapeptide



have been prepared in this manner in overall yields (based on the amount of C-terminal glycine on the starting resin) of 40% and 50%, respectively.

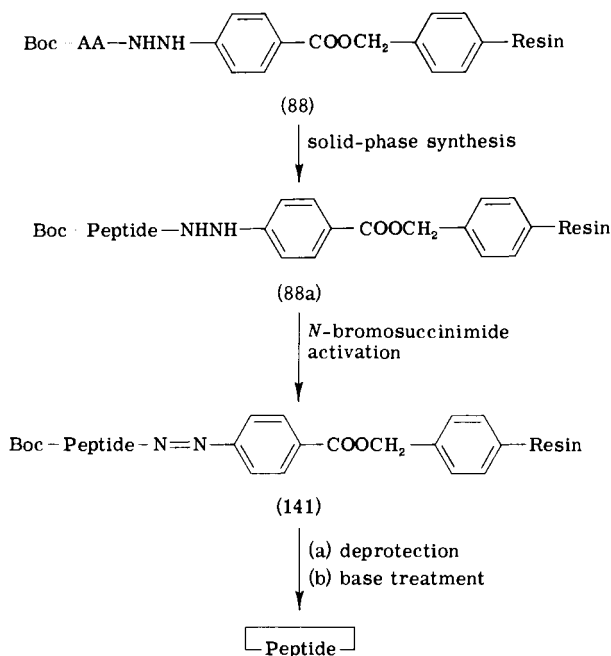
Table XXVI—Radioactivity Labeled Peptides Synthesized by the Solid-Phase Method

[¹⁴ C]-Oct-Ala-Abu-Arg	Benjamini <i>et al.</i> (1969)
[¹⁴ C]-Oct-Abu-Abu-Arg	Benjamini <i>et al.</i> (1969)
[¹⁴ C]-Oct-Ala-Gly-Arg	Benjamini <i>et al.</i> (1969)
[¹⁴ C]-Oct-Ala-Leu-Arg	Benjamini <i>et al.</i> (1969)
[¹⁴ C]-Oct-Ala-Ser-Arg	Benjamini <i>et al.</i> (1969)
[¹⁴ C]-Oct-Ala-Thr-Arg	Benjamini <i>et al.</i> (1969)
[¹⁴ C]-Oct-Abu-Thr-Arg	Benjamini <i>et al.</i> (1969)
[¹⁴ C]-Oct-Gly-Thr-Arg	Benjamini <i>et al.</i> (1969)
[¹⁴ C]-Oct-Leu-Thr-Arg	Benjamini <i>et al.</i> (1969)
└ Cys-[¹⁴ C]-Val	
Aad	Bauer (1970)
Phe-[¹⁴ C]-Gly-Gly	Smith <i>et al.</i> (1970)
Phe-Gly-[¹⁴ C]-Gly-Gly	Smith <i>et al.</i> (1970)
Phe-Gly-Gly-[¹⁴ C]-Gly-Gly	Smith <i>et al.</i> (1970)
[α,α- ³ H ₂]-Gly-Gly-Gly-Gly	Peters <i>et al.</i> (1970)
Gly-Gly-Gly-[α,α- ³ H ₂]-Gly	Peters <i>et al.</i> (1970)
[¹⁴ C]-Gly-Gly-Thr-Asn-Glu	Robinson <i>et al.</i> (1970)
[¹⁴ C]-Gly-Ala-Asn-Ala-Gly	Robinson <i>et al.</i> (1970)
[¹⁴ C]-Gly-Arg-Asn-Arg-Gly	Robinson <i>et al.</i> (1970)
[¹⁴ C]-Gly-Glu-Asn-Glu-Gly	Robinson <i>et al.</i> (1970)
[¹⁴ C]-Gly-Thr-Asn-Glu-Gly	Robinson <i>et al.</i> (1970)
[¹⁴ C]-Gly-Lys-Asn-Lys-Gly	Robinson <i>et al.</i> (1970)
[¹⁴ C]-Gly-Thr-Asn-Thr-Gly	Robinson <i>et al.</i> (1970)
[¹⁴ C-Ile ⁶]-Angiotensin II	Pisano (1969b)
[¹⁴ C-Pro ³ , ¹⁴ C-Pro ⁴]-Lysylbradykinin	Pisano (1969b)
[¹⁴ C-Pro ² , ¹⁴ C-Pro ³]-Bradykinin	Pisano (1969a)
[¹⁴ C ₁ -Gly ⁹]-Arginine-vasopressin	Walter and Havran (1971); Havran <i>et al.</i> (1971b)
[¹⁴ C ₁ -Gly ⁹]-Lysine-vasopressin	Walter and Havran (1971); Havran <i>et al.</i> (1971b)
[¹⁴ C ₁ -Gly ⁹]-Oxytocin	Walter and Havran (1971); Havran <i>et al.</i> (1971b)
([¹⁴ C]-Acetyl)-Thr-Thr-Ala-Glu-Thr-Leu-Asp-Ala-Thr	Benjamini <i>et al.</i> (1968a)
([¹⁴ C]-Acetyl)-Ala-Ala-Ala-Ala-Ala-Leu-Asp-Ala-Thr-Arg	Benjamini <i>et al.</i> (1968a)
([¹⁴ C]-Acetyl)-Thr-Thr-Ala-Glu-Thr-Leu-Asp-Ala-Thr-Arg	Benjamini <i>et al.</i> (1968a)
[¹⁴ C(U)-Leu ¹⁰]-Ile ⁵ -Angiotensin I	Thampi <i>et al.</i> (1968)
[¹⁴ C-Leu ¹¹]-Angiotensinogen	Skeggs <i>et al.</i> (1969)

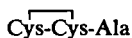
In order to effect solid-phase cyclization of [Gly⁵, Gly¹⁰]-gramicidin S, Sklyarov and Shashkova (1969) attached Boc-Orn-Leu-D-Phe-Gly-OMe through the δ-amino group of the ornithine residue to chloroform-oxymethyl polystyrene-2% divinylbenzene resin (Letsinger and Kornet, 1963; Letsinger *et al.*, 1964) and built up the decapeptide chain through six

cycles of standard solid-phase synthesis. Saponification of the C-terminal methyl ester, formation of the *N*-hydroxysuccinimide ester, *N*-deprotection and incubation in the presence of triethylamine gave resin-bound cyclic decapeptide. Removal of the solid support by HBr in trifluoroacetic acid afforded the fully active antibiotic in 20% overall yield.

Wieland *et al.* (1970) investigated peptide cyclization on the 4-carboxy-phenylhydrazide resin supports (88, 88a) through oxidative activation (141), deprotection and base treatment, but thus far only very low amounts of cyclic peptide could be obtained in this manner.



Intramolecular disulfide bond formation is a special type of cyclization requiring similar dilution conditions to minimize intermolecular dimerization or polymerization, and the solid-phase system has also been useful for this purpose. The disulfide containing tripeptide



has been prepared in 80% yield (Patchornik *et al.*, 1967; Bondi *et al.*, 1968) using cyclization on the Merrifield resin with the aid of ethylene diiodide (Weygand and Zumach, 1962) after the S-protecting trityl groups had been cleaved off by AgNO_3 in pyridine. The disulfide ring of oxytocin has been formed on the solid support after the nonapeptide was assembled and the S-protecting mercaptoethyl groups removed by thiophenol treatment

(Inukai *et al.*, 1968). It should be interesting to observe if these special exploitations of the solid-phase system will find more numerous applications in the future.

D. OTHER POLYMERIC PRODUCTS

The solid-phase concept is principally adaptable to any kind of repetitive synthetic procedure, and it is especially suited for the preparation of other polymeric substances such as polynucleotides or polysaccharides. The synthesis of oligonucleotides on solid supports has received wide application, and one recent report (Frechet and Schnerch, 1971) has been concerned with solid-phase synthesis of oligosaccharides. Oligonucleotide synthesis on polymer supports is more difficult than peptide synthesis since coupling yields are far from complete (50–80%) and sometimes quite low, and the demands on the selectivity of protecting groups are very high. Merrifield (1969a) has briefly reviewed the early efforts in this field. Recent contributions are those of Cramer and Köster (1968), G. M. Blackburn *et al.* (1969), Melby and Strobach (1969a,b), Kusama and Hayatsu (1970), and Potapov *et al.* (1971). Recent attempts to prepare oligoethylene terephthalates met with unsatisfactory results (Pénisson and Zahn, 1970).

VI. Comparison and Choice between Solid-Phase and Solution Synthesis

A. PRESENT STATE OF SOLID-PHASE SYNTHESIS

1. General Remarks

Several years ago the attitude of peptide chemists towards solid-phase peptide synthesis appeared to be rather definitive. Two opposing camps could be distinguished. One group comprised those investigators whose experience with solid-phase peptide synthesis made them enthusiastic believers. The other group consisted of rather uncompromising opponents, who objected either by principle or as a consequence of disappointing results obtained with the method. In the meantime a large number of methodological investigations and significant modifications have been published. An appraisal of the state of the art is necessarily more complicated. Comments and discussions about the subject have become considerably qualified. Moreover, some proponents from both groups have changed party.

A large number of peptides for biological studies have been prepared by solid-phase synthesis (see Tables XXII to XXV). The smaller ones (with up to ten or fifteen amino acid residues) may be purified to provide the desired compounds in homogeneous form by presently available fractionation procedures. Syntheses according to much larger target sequences

extending into the range of proteins give invariably complex mixtures. Although striking achievements have been made in fractionating nuclease preparations, the purified products are still at variance with the natural compounds with respect to biological potency. Presumably the solid-phase synthetic proteins contain considerable portions of deletion peptides that are, as yet, inseparable. Furthermore, very specialized procedures have been used in the nuclease purifications that will by their nature not be applicable to many proteins other than enzymes or enzyme inhibitors. The solid-phase procedure has recently been carried out through 187 cycles, and the most interesting methodological aspect of this achievement is the observation that amino acid incorporation apparently continued to function in high average yields throughout. Presumably it might continue to do so for longer sequences. Many methodological studies have now shown the occurrence of incomplete deprotection or incomplete coupling and deficiencies in removal of the solid support. Some of the factors influencing rate and degree of reactions have been recognized. Analytic procedures have been developed allowing some process controls, but the sensitivity needs to be considerably improved. Modified peptide to resin linkages and different solid supports have been studied with the aim of developing milder procedures for the final removal from the completed peptide. Finally, alternative solid-phase procedures have been devised and some show promising results. The volume of literature keeps growing at an increasingly rapid rate which should be considered the finest tribute to Merrifield's ingenious concept. It must also be noted that notwithstanding the numerous proposed modifications, the great majority of all work still follows essentially the standard experimental procedures developed by the inventor and his collaborators. Some of the principal requirements of the method are presently short of full experimental implementation, most importantly 100% complete reactions throughout. As a consequence, more methodological work is urgently needed and project-oriented applications must be subject to critical evaluation of product quality.

In the following discussion some of the above points will be dealt with in more detail, and a few selected examples shall serve to corroborate the conclusions.

2. Methodological Aspects

The solid-phase method has not been designed as just another approach to the synthesis of small peptides but for the express purpose of synthesizing proteins (Merrifield, 1969b). To achieve this aim, the experimental performance must approach the ideal requirements very closely. Let us consider the present state of the solid-phase procedure from this point of view. It appears that several basic prerequisites are well met by the method. (a) It

is *universal*, i.e., all amino acid residues that occur normally in proteins can be successfully incorporated into the growing peptide chain, including the "difficult" ones (tryptophan, histidine, methionine), provided special precautions are observed. (b) The method is *rapid*, allowing extension of the peptide chain by one to six amino acid residues per day. Programmed mechanized machines have been developed for full-time operation. (c) The procedure is *easy and convenient* to perform and does not require highly specialized knowledge of preparative synthetic organic procedures. (d) The overall efficiency in individual peptide bond formation does not seem to diminish in proportion with increasing chain length, as judged from the few protein syntheses so far published.

These assets are presently offset by four main deficiencies. (a) *Failures of quantitative incorporation* of each amino acid residue can be caused by incomplete coupling or by less than quantitative cleavage of $N\alpha$ -protecting groups. The latter shortcoming might occur as frequently as the former, but both are influenced by (i) the sequence, (ii) the steric properties of reactants, (iii) the solvent polarity, and (iv) the degree of resin-cross-linking. (b) Racemization can occur during the coupling reaction; and *side reactions* due to insufficient stability of side-chain protection have been observed. Improved combinations of $N\alpha$ - and side-chain protecting groups are already being examined. Minimization of racemization will require special reaction control (Kemp, 1973); which could hardly improve matters for those who fail to purify their protected amino acids to better than 99% optical purity (see Kemp *et al.*, 1970a). (c) The benzyl ester *peptide to resin linkage* is inadequate in two respects, (i) incomplete stability during deprotection causing loss of peptide during synthesis, and (ii) incomplete cleavage of the final product from the solid support enforcing the use of destructive conditions. (d) Available *analytic controls* are not sensitive enough to determine the reaction progress in the 99.5–99.9% range.

Several of these deficiencies could probably be eliminated or minimized through the application of presently available methodology. More complete reactions might be obtainable in solvents, such as toluene (Kemp, 1973), or with the use of surface bonding of peptide to solid support (Potts *et al.*, 1971; Bayer *et al.*, 1970c). Side reactions could be minimized by the use of more stable side-chain protection such as ring substituted benzyl derivatives or trifluoroacetyl or fluorenylmethyloxycarbonyl groups. Racemization might be lowered by working at 0° in favorable solvents. Solid supports such as cellulose might offer the possibility of very mild final removal by selective enzymic degradation of the support. Peptide to resin linkage through one or several D-amino acid residues might be split by proteolytic enzymes that specifically cleave such bonds (e.g., enzymes of microbial ori-

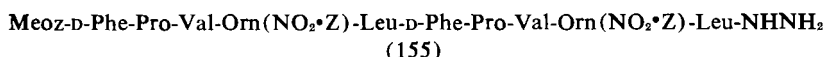
gin). Loss of peptide during a synthesis can be minimized by using α -amino protecting groups [e.g., the 2-(*p*-biphenyl)isopropoxyloxycarbonyl group] which are removable under milder conditions than those required for complete cleavage of the *tert*-butyloxycarbonyl group.

3. Product Purity

At present solid-phase synthesis does not produce homogeneous peptides because (a) reactions do not proceed to 100% completion, (b) side-chain protection is not sufficiently stable, and (c) trace racemization can occur. Peptide chemists, however, have always aimed at products of single molecular nature. In solution synthesis this is attempted through painstaking purification after each peptide bond formation. In solid-phase synthesis, on the contrary, removal of side product contamination is postponed until after removal of the solid support. Therefore, the accumulated contaminants form a complex mixture, and purification becomes so extremely difficult that in some cases large multiples of the time required for the synthesis have to be spent on purification. The central question at the present time is: Which solid-phase synthetic peptide can be purified to homogeneity and which cannot, by available fractionation methods? The answer depends on size, composition, and sequence which determine such critical properties as stability, solubility, and charge. The answer also depends to no small measure on the experience and proficiency of the individual investigator with respect to peptide fractionation. It is probably safe to say that through determined efforts at purification homogeneous material should be obtained from many solid-phase syntheses of up to decapeptides. Examples of apparently homogeneous final products in this range of size include such biologically active peptides as: thyrotropin releasing hormone, melanotropin release inhibiting factor, Ile⁵- and Val⁵-angiotensin II, oxytocin and analogs, arginine- and lysine-vasopressin and analogs, bradykinin, antamanide and analogs, gramicidin S and analogs.

At the time of completing this review an "improved" solid-phase synthesis of gramicidin S was published by Izumiya and collaborators (Ohno *et al.*, 1971) which might be considered as the most perfect execution of the method. The *p*-methoxybenzyloxycarbonyl group was used for α -amino group protection, the *p*-nitrobenzyloxycarbonyl group for the protection of side-chain amino functions. Leucine served as the C-terminal amino acid which was linked to chloromethylated copolystyrene-2% divinylbenzene resin. Deprotection was carried out with 1 *N* hydrogen chloride in acetic acid, and coupling with 2.5 equivalents of protected amino acids and dicyclohexylcarbodiimide in methylene chloride for 4-8 hours. The completed decapeptide was re-

moved from the solid support by hydrazinolysis giving 4.1 gm (97%) of crystalline product (155) which was judged to be 98% pure by absorption at 268 nm (*p*-nitrobenzyloxycarbonyl group absorption). This amounts to an average of 99.4% yield in each coupling. In this synthesis several improvements were made over the standard solid-phase procedure. The *N* α -*p*-methoxybenzyloxycarbonyl group was obviously completely cleaved in each cycle; the *N* δ -*p*-nitrobenzyloxycarbonyl group of the ornithine residues was completely stable throughout the synthesis; the cleavage of the final product from the resin by hydrazinolysis was mild and practically quantitative.



Solid-phase syntheses of undeca- to eicosapeptides can, at present, provide homogeneous products in favorable cases where long experience of the investigator with the particular peptide should aid in highly efficient fractionation of the synthetic conglomerate. Examples include α -MSH, gramicidin A and B, and α -ACTH-(1-19). The investigator claiming homogeneity for peptides in this range of size must, however, grant to his peers the privilege of doubt. Only a series of independent and highly sensitive analytic criteria can demonstrate molecular uniformity with some certainty. For reasons unknown, some of the most sensitive tests for peptide homogeneity seem to have not yet been applied to products of solid-phase synthesis, such as dansyl end-group determination (for a recent review, see Seiler, 1970) or SDS-gel electrophoresis. Two dimensional chromatography-electrophoresis (peptide mapping) and enzymatic purity tests (Zuber, 1960) have been applied only in a few instances; polyacrylamide gel electrophoresis has been used once (Blake *et al.*, 1972).

Peptides larger than eicosapeptides can hardly be obtained in uniform state by the present experimental execution of the solid-phase method. This will require either improved synthetic procedures or more powerful fractionation methods or both. As described in Section III,F,2, such purification methods as countercurrent distribution (which should be used more frequently), ion exchange chromatography, combined partition and gel chromatography, affinity chromatography, functional purification (specific enzymic treatment) and peptide mapping have been used for products prepared by solid-phase synthesis. Other recently developed methods which might possess even higher fractionation power such as rotating free zone electrophoresis (Hjertén, 1970), isoelectric focusing in pH gradients (Haglund, 1971) or in polyacrylamide gel (Finlayson and Chrambach, 1971), helix countercurrent chromatography (Ito and Bowman, 1971), or some fu-

ture form of affinity electrophoresis might merit investigation of their performance with solid-phase synthetic peptides.

There can be little doubt that, at present, a solid-phase peptide synthesis has to be followed up by extensive product purification and that the size limit for homogeneous peptides lies at approximately decapeptides with possible favorable exceptions up to eicosapeptides.

4. Scope and Limitations

Does the limitation to decapeptides and exceptionally to eicosapeptides apply generally? I suppose it does not. As mentioned above, the solid-phase method has been designed for protein synthesis. The critical question whether this will be within the scope of the method has already been tested in part. Conceivably it was to be expected that the efficiency of incorporation of amino acid residues would gradually decrease with increasing chain length and eventually become too minute for practical purposes. In other words, one might foresee an increasing number of chain terminations with increasing size of the synthetic peptide resulting in zero amino acid incorporation at some critical size. The nuclease and growth hormone syntheses have provided a most significant methodological information. It appears that the coupling efficiency remains surprisingly high up to 123 cycles or up to 187 cycles. If there should be any limitation, it must reside with longer chain lengths. Thus the method appears to remain highly functional up to at least 200 amino acid residues, which corresponds to a medium-sized protein.

Certainly, the products that have so far been prepared are incredibly complex mixtures, even after purification. However, some biological problems can also be studied with the use of such mixtures. At present there is really no alternative solution method available which is capable of producing protein preparations in more than microgram quantities.

Thus, the present limitation of the solid-phase method does not reside in an upper size restriction. Mixtures of protein can readily be prepared. One has to decide in each case how useful mixtures (after purification!) might be for the study of biological or biochemical questions or which correlation to natural products can be deduced.

It is hoped that future improvements of the present synthetic procedure will reduce the product complexity. At the present state of development *average* coupling yields are mostly below 99%. It should be possible to develop within the next few years by known organic chemical methodology a procedure providing 99% average yields per cycle. This will be a good step forward in the direction of obtaining a future solid-phase method featuring a desirable 99.9% average coupling efficiency.

B. PRESENT STATE OF SOLUTION SYNTHESIS

1. Reviews

Peptide synthesis has been thoroughly discussed periodically. The main, comprehensive reviews are those of Fruton (1949), Grassmann and Wünsch (1956), Goodman and Kenner (1957), Greenstein and Winitz (1961), Hofmann and Katsoyannis (1963), Schröder and Lübke (1965, 1966), and Bodanszky and Ondetti (1966). Several didactic books have recently appeared that contain concise essentials of the field; among them those by Kopple (1966), Elmore (1968), Jakubke and Jeschkeit (1969), and Law (1970). A table of synthetic peptides has been published (Pettit, 1970, 1971). Specialist Periodical Reports, edited by Young, are now available (Young, 1969, 1970, 1971). The proceedings of the European, Japanese, and American Peptide Symposia reflect in lively manner the progress and problems in peptide synthesis during the past 15 years.* The following brief account shall touch upon a very few selected examples of recent developments in protecting groups and methods for peptide bond formation. The state of the art will be illustrated by a brief discussion of two recent synthetic achievements, glucagon and ribonuclease A S-protein.

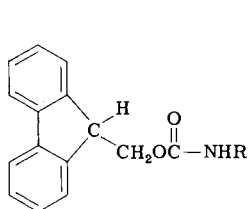
2. Protecting Groups

Recent developments in amino group protection include the 2-(*p*-biphenyl)isopropylloxycarbonyl group (Sieber and Iselin, 1968a,b) which has been discussed in Section III,C,1. The recently announced 9-fluorenylmethoxycarbonyl group (102) of Carpino and Han (1970)† is completely stable to acid treatment and to hydrogenolysis; it is removed by simply dissolv-

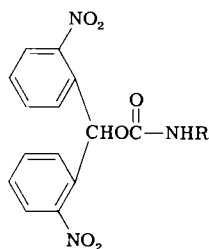
* Publications of European Peptide Symposia: 1st (Prague, 1958): *Collect. Czech. Chem. Commun.* **24** (Spec. Issue), pp. 1–160 (1959); 2nd (Munich, 1959): *Angew. Chem.* **71**, pp. 741–743 (1959); 3rd (Basel, 1960): *Chimia* **14**, pp. 366–380 (1960); 4th (Moscow, 1961): *Collect. Czech. Chem. Commun.* **27**, pp. 2229–2262 (1962); 5th (Oxford, 1962): Young (1963); 6th (Athens, 1963): Zervas (1966); 7th (Budapest, 1964): Bruckner and Medzihradsky (1965); 8th (Noordwijk, 1966): Beyerman *et al.* (1967a); 9th (Orsay, 1968): Bricas (1968); 10th (Abano, Terme, 1969): Scoffone (1971); 11th (Vienna, (1971): Nesvadba (1973). Publications of the American Peptide Symposia: 1st (New Haven, Connecticut, 1968): Weinstein and Lande (1968, 1970); 2nd (Cleveland, Ohio, 1970): Lande (1972); 3rd (Boston, 1972): Meienhofer (1972). The proceedings of some of The Japanese Symposia, 1st (1962) to 9th (1971), have been published in Japanese by the Protein Research Foundation, Osaka.

† 9-Fluorenylmethyl chloroformate, is commercially available from Aldrich Chemical Company, Inc., Milwaukee, Wisconsin.

ing the peptide in liquid ammonia or by treatment with ethanolamine or morpholine in organic solvents.



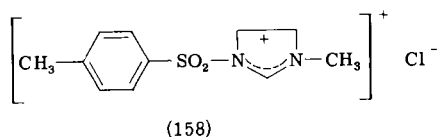
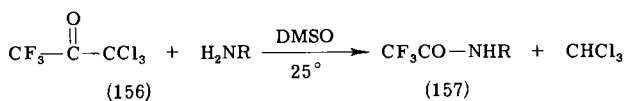
(102)



(103)

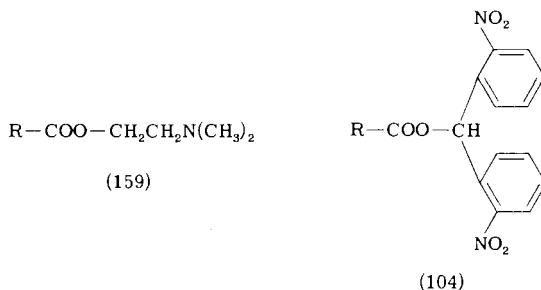
The 2,2'-dinitrodiphenylmethyloxycarbonyl group (103) can be cleaved photolytically at $\lambda > 3200 \text{ \AA}$, thus introducing a new, highly selective deprotection procedure (Patchornik *et al.*, 1970).

For some of the commonly used amine protecting groups, improved procedures for their introduction into amino acids or peptides have been developed. *tert*-Butyloxycarbonyl fluoride (Schnabel *et al.*, 1968a,b,c, 1971a) reacts 5–10 times faster than the azide, thus greatly facilitating the preparation of Boc-amino acids as described in Section III,C,3. Introduction of the trifluoroacetyl group (157) with the use of unsymmetrical trichlorotrifluoroacetone (156) proceeds under neutral conditions producing chloroform as the only by-product (Panetta and Casanova, 1970). The formyl group is easily introduced by a carbodiimide condensation (Thomas, 1967), and a water-soluble reagent for amine tosylation, *N*-tosyl-*N'*-methylimidazolium chloride (158) (Jozefczak *et al.*, 1970), would appear to have certain advantages over the commonly used tosyl chloride which often requires mixtures of aqueous and organic solvents. A recent report about an efficient electrolytic cleavage of the *N*-*p*-toluenesulfonyl group (Okumura *et al.*, 1971) promises to enhance the applicability of this valuable protecting group. Interesting new developments in carbonyl group protection include: the β,β -dimethylaminoethyl ester (159) by Greben *et al.* (1968), which is stable to

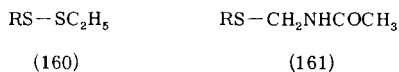


(158)

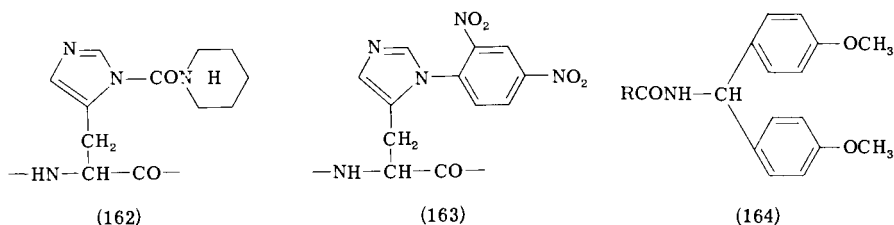
hydrogen bromide in acetic acid and removed by 2–4% NaHCO_3 in dimethylformamide–water; the *p*-methylthiophenyl ester by Johnson and Jacobs (1968a,b) which has been discussed in detail in Section III,B,2 and the 2,2'-dinitrodiphenylmethyl ester (104) by Patchornik *et al.* (1970) which is cleaved by photolytic treatment ($\lambda > 3200\text{\AA}$).



Many promising new groups have been developed for side-chain protection. Among those for cysteine thiol protection are the ethylmercapto group (160) of Inukai and collaborators (1967) which is cleavable by mercaptans and stable towards acid and base, and the acetamidomethyl group (161) developed by Veber *et al.* (1968). This latter group (161) is stable to acids (trifluoroacetic acid, hydrogen bromide in acetic acid, hydrogen chloride, hydrogen fluoride at 0°) and to acidic and alkaline aqueous solutions in the pH range 0–13 at 25° . It is easily cleavable by mercuric ions at pH 4 and room temperature. Most importantly, the solubility characteristics of the acetamidomethyl group allow its use in both organic solvents and aqueous media.



For the protection of the imidazole moiety of histidine, the piperidinocarbonyl group (162) has been described (Jäger *et al.*, 1968). It is stable to acid treatment, but easily removed by hydrazine or sodium hydroxide. The 2,4-dinitrophenyl group (163) has been used by Shaltiel (1967) and Shaltiel and Fridkin (1970) for protection of the histidine imidazole function, featuring highly selective cleavage by thiols and complete stability to acid. It has proven its value in several syntheses. To avoid undesired side reactions caused by the amide side-chain functions of glutamine and asparagine residues (e.g., nitrile formation in carbodiimide mediated peptide coupling), the 4,4'-dimethoxybenzhydryl group (164) has been introduced by König and Geiger (1970b). While stable to catalytic hydrogenation, hydrazinolysis, and base, it can be removed by trifluoroacetic acid, hydrogen bromide in acetic acid, or hydrogen fluoride.



The availability of these and many other newly developed protecting groups has increased the flexibility and versatility of strategy in peptide synthesis. A very important contribution has been made to the protection-deprotection problem by Sakakibara's discovery of the effectiveness of anhydrous liquid hydrogen fluoride for rapid and quantitative removal of many commonly used protecting groups (Sakakibara and Shimonishi, 1965; Sakakibara, 1971) (Table XXVII). The applications of this procedure to the cleavage of the peptide to resin linkage in solid-phase synthesis have been discussed in Section III,E,1.

Undoubtedly, more highly selective protecting groups and other useful reagents are still required. There is a particular need for groups which would confer to peptides increased solubility in organic solvents or in aqueous systems.

The extent to which side-chains need to be protected depends very much on the peptide to be synthesized and on the condensation methods to be used. Frequently, some of the less reactive amino acid side-chain functions, such as those of serine, threonine, tyrosine, or histidine can be left unpro-

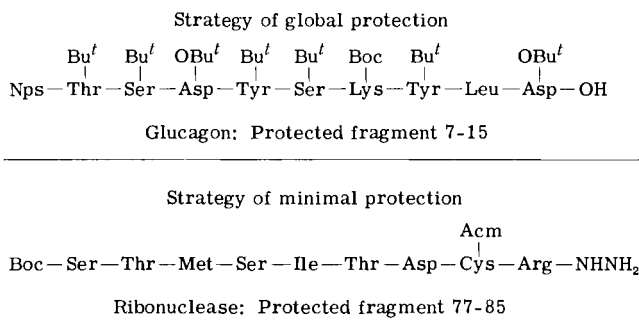


FIG. 25. Strategies of global and minimal protection of amino acid side-chain functions. (Top, from Wünsch *et al.*, 1968a; bottom from Jenkins *et al.*, 1969).

Table XXVII—Behavior of Various Protecting Groups in Hydrogen Fluoride in the Presence of Anisole^a

Protecting Group	Stability ^b	Conditions	
		Temp (°C)	Minutes
<i>tert</i> -Butyloxycarbonyl	A	0	<30
<i>tert</i> -Amyloxycarbonyl	A	0	<30
Benzyloxycarbonyl	A	0	<30
<i>p</i> -Methoxybenzyloxycarbonyl	A	0	<30
<i>p</i> -Nitrobenzyloxycarbonyl	C	20	60
<i>p</i> -Chlorobenzyloxycarbonyl	A	0	60
<i>N</i> α or <i>N</i> ϵ -Tosyl	C	20	120
<i>N</i> θ -Tosyl (Arg)	A	0	30
<i>N</i> ^{4m} -Tosyl (His)	A	0	<30
<i>N</i> θ -Nitro	A	0	<30
Trifluoroacetyl	C		
Formyl	C	20	120
Phthalyl	C	20	120
<i>o</i> -Nitrophenylsulfenyl	A	0	<30
Methyl ester	C	20	120
Ethyl ester	C	20	120
<i>tert</i> -Butyl ester	A	0	<30
Benzyl ester	A	0	30
<i>p</i> -Nitrobenzyl ester	B (15)	20	60
<i>O</i> - <i>tert</i> -Butyl	A	0	<30
<i>O</i> -Benzyl (Ser, Thr)	A	0	<30
<i>O</i> -Benzyl (Tyr)	B (70)	0	60
<i>S</i> -Ethyl	C	20	120
<i>S</i> - <i>tert</i> -Butyl	A	20	60
<i>S</i> -Benzyl	A	20	30
<i>S</i> - <i>p</i> -Methoxybenzyl	A	0	<30
<i>S</i> -Diphenylmethyl	A	0	30
<i>S</i> -Trityl	A		
<i>S</i> -Acetamidomethyl	C	0	
Merrifield resin ester	A	20	30–60

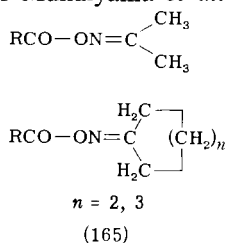
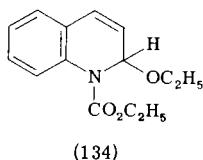
^a From Sakakibara, 1971.^b A, Removable; B, partially removable; C, stable. Number in parentheses refer to percent removal.

tected, for example in azide or some active ester condensations. However, for the synthesis of glucagon Wunsch (1967) found through much experimentation that a strategy of “*global protection*” was required for a successful synthesis of this hormone. Global protection, i.e., protection of all side-chain functions, provides maximal safety against undesired side reactions and flexibility in the choice of coupling methods. On the other hand, the

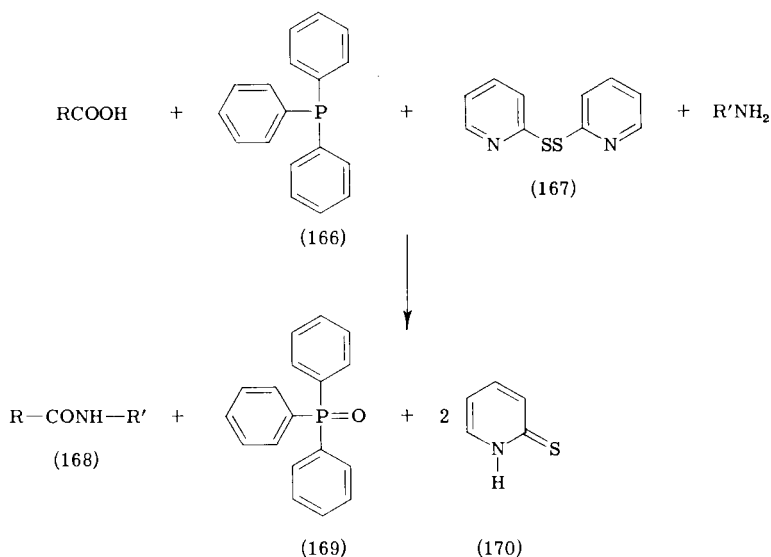
presently used protecting groups create problems with larger peptides by decreasing their solubility in organic solvents. Therefore, Hirschmann and Denkwalter and their collaborators reduced the use of blocking groups to a minimum in their synthesis of the S-protein portion of ribonuclease A (see Hirschmann and Denkwalter, 1970). "*Minimal protection*" does, however, restrict the choice of peptide condensation methods. Fig. 25 illustrates global and minimal protection taking two nonapeptides as examples, each of which possess eight amino acid residues with functional side-chains.

3. Peptide Bond Formation

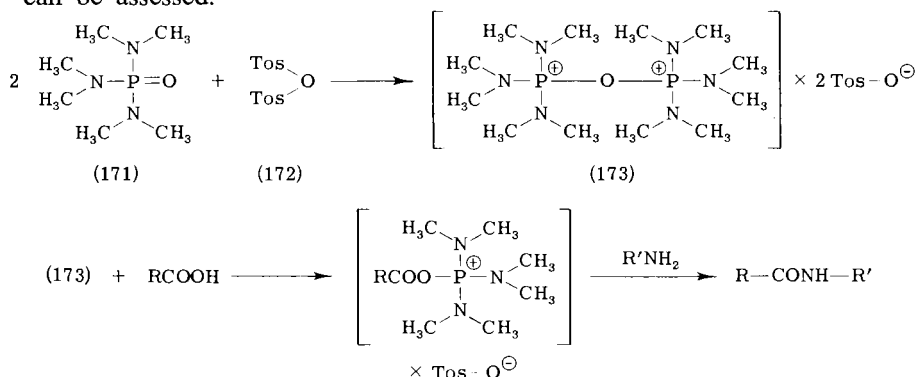
Four methods of peptide bond formation proved to be powerful enough to find general use and applicability in peptide synthesis. The azide, mixed anhydride, carbodiimide, and active ester methods remain essentially unchallenged in this position over the past 15–20 years through hundreds of peptide syntheses. Most of the recent progress can be attributed to further improvements, refinements and expansion of these four methods rather than to the development of other new methods. Thus, the scope of the *active ester method* has been enlarged by many modified esters, in particular those providing anchimeric catalysis, such as hydroxyquinoline esters (Jakubke and Voigt, 1966), catechol esters (Jones and Young, 1967, 1968), 2-pyridyl thiol esters (Lloyd and Young, 1968, 1971), and 2-pyridyl esters (Dutta and Morley, 1971, 1973). Suppression of acylurea formation and lowering of racemization in *carbodiimide mediated coupling* was achieved by addition of *N*-hydroxysuccinimide (Wünsch and Drees, 1966; Weygand *et al.*, 1966) or by 1-hydroxybenzotriazole derivatives (König and Geiger, 1970a). Essentially racemization-free coupling of peptides (less than 0.2% diastereoisomer formation) can be obtained by the *mixed anhydride procedure* using the Anderson modification (Anderson *et al.*, 1967). New developments of reagents for peptide bond formation which appear to be interesting and promising include *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (134) (Belleau and Malek, 1968). This condensing reagent, like carbodiimide, is simply added to the mixture of the carboxyl and amine components. Ketoxime esters (165) which had been known for years as very slowly reacting substances (Losse *et al.*, 1964) were found to form peptide bonds very fast and efficiently with the aid of acid catalysis (Fujino and Nishimura, 1969). In the oxidation-reduction condensation of Mukaiyama *et al.* (1970), tri-



phenylphosphine (166) and 2-thiopyridine disulfide (167) are used for peptide bond (168) formation, leaving triphenylphosphine oxide (169) and 2-thiopyridine (170) as by-products.



Kenner's acyloxyposphonium salts (173) are prepared from hexamethylphosphoramide (171), a powerful solvent for large protected peptides, and from tosic anhydride (172). The method has yielded excellent results in fragment condensations of protected peptides (Gawne *et al.*, 1969). An octapeptide was obtained in 81% yield from a benzyloxycarbonyl pentapeptide and a tripeptide ester, another one in 75% yield (2 + 6 condensation). These new methods showed promising results in the hands of the authors, but further work will have to be awaited before their general usefulness can be assessed.



A risk of *racemization* is always involved when peptide bonds are formed between optically active amino acids. Since biological activity of peptides and proteins is dependent on their optical purity, racemization has to be carefully avoided. To be useful, a synthetic method must proceed with no more than minimal racemization. Reaction conditions under which the danger of racemization is minimal have been developed for all four generally used methods discussed above. The azide method had long been considered to be *racemization-free*, but the recent development of a highly sensitive racemization test through isotopic dilution, which measures in the ± 0.001 to ± 0.05 sensitivity range (Kemp *et al.*, 1970a,b), led to the recognition that traces of racemization always occur. Recently, as much as 20% racemization was detected after a fragment condensation by the azide method during a synthesis of calcitonin (Sieber *et al.*, 1970; Riniker *et al.*, 1972). It has since become clear and generally acknowledged* that a "*racemization-free*" method does not exist. It will be necessary to optimize coupling conditions on the basis of known solvent and temperature effects (Goodman and McGahren, 1966; Goodman and Glaser, 1970; Kemp *et al.*, 1970b; Kemp, 1973) in every individual peptide condensation, that is of critical importance during syntheses of large peptides.

Several procedures (tactics) aimed at providing rapid peptide synthesis in solution have been developed. In these schemes solvent extraction (washing) procedures are used for the work-up after the coupling, and any further purification of intermediates is omitted. Excess acylating component (carboxyl component and reagent) is used to achieve complete or near complete peptide bond formation. Water-soluble carbodiimides (Sheehan *et al.*, 1965), Boc-amino acid *N*-hydroxysuccinimide esters (Laufer and Blout, 1967), and the carbonic carboxylic mixed anhydride method have been used (Tilak, 1970) for this purpose. *N*-Carboxyamino acid anhydrides were employed for the rapid controlled synthesis of peptides in aqueous medium (Denkewalter *et al.*, 1966).

4. Selected Examples

Since Schröder and Lübke (1966) reviewed synthetic achievements several outstanding peptide syntheses have been accomplished. The impressive synthesis by the Merck group of the S-protein part of bovine ribonuclease A containing 104 amino acid residues will be briefly described below. Two syntheses of adrenocorticotropin, possessing thirty-nine amino acid residues, have been achieved. Porcine ACTH has been assembled by Schwyzzer and Sieber (1963), and Bajusz and Medzihradszki prepared the human hor-

* Forum discussion at the Eleventh European Peptide Symposium, Vienna, 1971.

hormone (Bajusz *et al.*, 1967). Very interesting and commercially important are syntheses of highly potent N-terminal fragment analogs: [β -Ala¹]-ACTH-(1-17) 4-amino-*n*-butylamide (Geiger, 1971), [D-Ser¹, Lys¹⁷, Lys-NH₂¹⁸]- β -ACTH-(1-18) (Desaulles *et al.*, 1969), and [D-Ser¹, Nle⁴, Val-NH₂²⁵]- β -ACTH-(1-25) (Boissonnas *et al.*, 1966). These peptides exhibit 7-8 times higher biological activity than the natural hormone. Several calcitonin syntheses have been reported: porcine calcitonin (Rittel *et al.*, 1968; Riniker *et al.*, 1969; Guttmann *et al.*, 1968); human calcitonin (Sieber *et al.*, 1968); salmon calcitonin (Guttmann *et al.*, 1969). The efforts of many years have been crowned by a successful synthesis of glucagon, which will be outlined below; and secretin has been synthesized (Bodanszky *et al.*, 1966, 1967a) by several routes comparing the strategies of incremental chain elongation (Bodanszky *et al.*, 1967b) and of fragment condensation (Ondetti *et al.*, 1968b).†

Presently, syntheses of ribonuclease T₁ (104 amino acid residues) (Hofmann, 1973; Beacham *et al.*, 1971; Yanaihara *et al.*, 1969), of staphylococcal nuclease-(6-47) (Zeiger and Anfinsen, 1972) and of proinsulin (Zahn *et al.*, 1973; Yanaihara *et al.*, 1970) are in progress.

The synthesis of bovine ribonuclease A-(21-124) by Hirschmann, Denkwalter, and collaborators (Denkwalter *et al.*, 1969; Strachan *et al.*, 1969; Jenkins *et al.*, 1969; Veber *et al.*, 1969; Hirschmann *et al.*, 1969) introduced into peptide synthesis several innovative experimental procedures: (a) a new rapid method for peptide bond formation, "controlled" sequential *N*-carboxyanhydride couplings in aqueous phase, (b) a new S-protecting group, the acetamidomethyl group, which confers excellent solubility upon cysteine-containing peptides, (c) a new strategy, minimal side-chain protection (see Fig. 25) (Denkwalter and Hirschmann, 1969; Hirschmann and Denkwalter, 1970). The azide method was used for the condensation of the nineteen fragments which had mostly been prepared by the sequential *N*-carboxyanhydride method. Finally two large fragments were obtained containing forty and sixty-four amino acid residues. Their condensation by the azide method proceeded with very low yield. The S-peptide was added to the completed synthetic S-protein for generation of ribonuclease activity (Richards and Vithayathil, 1959). Very small amounts of synthetic material have so far been obtained, and preparations of larger amounts are still in progress. The condensation of the larger fragments still presents the most difficult problem. This will be one of the major general obstacles for solution synthesis of large peptides or proteins in the years ahead.

The glucagon synthesis of Wünsch and collaborators (Wünsch, 1967;

† See Chapter 2 by Bodanszky, A., for a discussion on secretin synthesis.

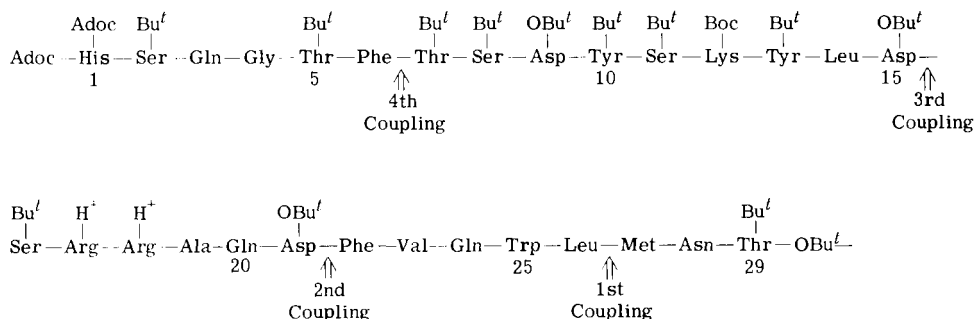


FIG. 26. Protected nonacosapeptide intermediate of a glucagon synthesis by solution methods (Wünsch, 1967). Arrows indicate points of fragment condensation.

Wünsch and Wendlberger, 1967; 1968; Wünsch *et al.*, 1968a-d) is an impressive example of a carefully executed synthesis in which undesired side reactions were minimized through a strategy of global side-chain protection. The protected nonacosapeptide, shown in Fig. 26, was prepared by fragment condensation from five intermediates, starting with the C-terminal tripeptide intermediate. All four condensations were carried out with the dicyclohexylcarbodiimide-*N*-hydroxysuccinimide procedure (Wünsch and Drees, 1966; Weygand *et al.*, 1966). Deprotection with trifluoroacetic acid followed by gel filtration on Sephadex G-50 gave chromatographically homogeneous glucagon, which crystallized from aqueous solution at pH 9.3 (Fig. 27). The synthetic hormone was indistinguishable from the natural hormone in biological potency, and in physical and chemical characteristics. The synthesis proved the structure established by Bromer *et al.* (1956) to be correct. Furthermore, a quantity of about 5 gm of crystalline hormone was synthesized. Since glucagon is only sparingly available from natural sources, the synthesis made this hormone available for biological and clinical studies.

5. Scope and Limitations

During the past 15 years an impressive number of peptides with up to forty amino acid residues have been prepared by solution methods. The main effective coupling methods had been developed by 1955, i.e., the azide, mixed anhydride, carbodiimide, and active ester methods. Active esters are most frequently used. The number of applications surpasses those of the other three methods combined. Progressively more effective ways and means for the formation of peptide bonds are derived from refinements and improvements of the four methods. Optimal reaction conditions have been elaborated in efforts to minimize both racemization and undesired side reac-



FIG. 27. Crystals of synthetic glucagon. (1500:1). From Wünsch *et al.* (1968c).

tions. New methods have not expanded the scope of peptide synthesis to any significant degree.

A class of most useful protecting groups, those based on the *tert*-alkyl moieties, is of relatively recent origin. The combination of their use with that of protecting groups derived from the benzyl group has been most successful in the majority of recent syntheses. Promising new protecting groups have been developed, but there is a need for even more.

The strategy of incremental (stepwise) chain elongation (see Fig. 2B) has recently been used through twenty-six stages in a synthesis of a heptacosapeptide (secretin). Fragment condensation (see Fig. 2A) has served to assemble still larger sequences (see Table II).

How far can peptide synthesis in solution be expected to go? Or, in other words, what are the prospects of protein synthesis by solution methods? The main obstacles foreseeable for condensations of large peptide fragments are the same as those that have impeded past syntheses. These are (a) insolubility of large protected peptides in organic solvents, (b) low reaction rates, (c) increased danger of racemization, (d) increased difficulties in purification and analytic characterization, and (e) very large work load.

These present shortcomings illustrate the need for further methodological developments. It appears to be unlikely that useful quantities (>10 – 100

mg) of large peptides containing over fifty to eighty amino acid residues could be prepared by presently available methods of solution synthesis. Eventually the scientific merit of a synthesis for its own sake will decrease (unless some striking new methodology is applied). The chemical synthesis of proteins will be motivated by the need for producing products for biological, biochemical, or medical research that cannot be obtained from natural sources. The proof of a protein structure by synthesis will not be a prime general concern because the limits of uncertainty are similar to those of sequence analysis*; and x-ray diffraction has now provided an effective alternative (Drenth *et al.*, 1968).

Before proteins can be synthesized in some routine fashion, we must develop (a) protecting groups that increase the solubility of peptides and/or more powerful solvents, (b) accelerated peptide bond formation by catalysis or new types of condensation procedures, (c) means to eliminate undesired diastereoisomers from products, (d) more powerful fractionation and analytic procedures (although the present methods are still reasonably adequate especially when used in combination). (e) It would not be appropriate to predict or promise any reduction in work load!

C. PROJECT-ORIENTED CHOICE OF METHOD

1. *Comparison of Solid-Phase and Solution Synthesis*

Evaluation of the advantages and disadvantages of solid-phase and solution synthesis reveals an inverse cross-correlation almost point by point (Table XXVIII). Solid-phase synthesis is very fast and convenient, while solution synthesis is slow and laborious. Solution synthesis of larger peptides is impeded by problems of low solubility, failures of fragment condensation, and danger of considerable racemization. These difficulties do not exist in solid-phase synthesis. In fact, the elegant elimination of the grave problems of low solubility faced by solution synthesis is one of the most important innovations of the solid-phase method, although this was probably not a prime consideration when it was designed. Operating a solid-phase synthesis does not require special organic chemical experience, while a very high requirement of such expertise has been one of the limitations in solution synthesis, and it has prevented the field in the past from becoming more closely integrated with other areas of research.

Solid-phase synthesis (Table XXVIII), on the other hand, produces peptides that are mostly microheterogeneous mixtures which cannot be defined by presently available analytic methods and which require intensive

* However, the structure proof by synthesis is sufficiently accurate for fragments obtained by enzymic digestion of the protein. This has been performed, for example, by Young *et al.* (1968a) with tobacco mosaic virus protein, sequence 42-46.

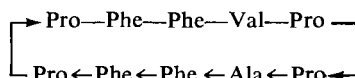
Table XXVIII—General Features of Solid-Phase and Solution Synthesis of Peptides

Solid-phase synthesis	Solution synthesis
<i>Favorable features</i>	<i>Favorable features</i>
Speed	Product: Homogeneous
No solubility problem	Adequate analysis
General synthetic experience sufficient	Purification of intermediates
Automation possible	<i>Unfavorable features</i>
<i>Unfavorable features</i>	Slow, laborious
Product: mixture	Insolubility
a. <100% Cleavage of N-protection	Failure of fragment condensation
b. <100% Peptide bond formation	Racemization danger
c. Small amount of racemization (0.1%)	Much special experience required
Removal from solid phase	
a. Incomplete	
b. Product degradation	
Extensive product purification needed	
No adequate analysis (0.1–0.5%)	

purification without assurance of achieving product homogeneity. Solution synthesis, however, affords generally homogeneous peptides because intermediates are usually purified after each peptide bond formation. Analytic procedures are mostly adequate even for quite large peptides, because they also are applied after each condensation step. In solution synthesis reaction mixtures usually consist of few (sometime predictable) components.

The comparison in Table XXVIII clearly shows that the strength of solid-phase synthesis lies in *operational efficiency*. The strength of the solution methods lies in *product quality*. Therefore, in cases where identical peptides of equal purity can be obtained through either approach, the solid-phase procedure offers the advantages of time saving, easy manipulation, and, frequently, of higher overall yields* (see, also, Wünsch, 1971).

Direct comparisons of the performance of both approaches in syntheses of identical peptides have been made for a variety of systems. Two typical examples are syntheses of antamanide and hepta- γ -glutamic acid. The cyclic decapeptide antamanide (174), the antidote to the fungal toxin amanitin (Wieland and Gebert, 1966; Wieland, 1968) was first synthesized by solu-

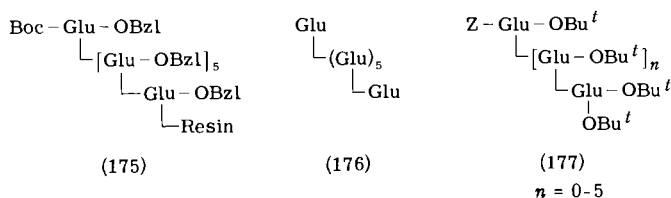


(174)

* Yields are based on the amount of C-terminal amino acid. In solid-phase synthesis the excesses of reactants and reagents are not taken into account; see discussion by Bodanszky (1970).

tion methods (Wieland *et al.*, 1968, 1969c). A subsequent preparation of antamanide by solid-phase synthesis (Wieland *et al.*, 1969a) afforded a 98% yield of the linear decapeptide intermediate. The purification of the cyclic product was greatly facilitated by the ease of crystallization of antamanide. Thus solid-phase synthesis gave a higher yield of homogeneous product and required a much smaller effort than solution synthesis.

In another comparative study, a solid-phase synthesis using benzyl ester protection (175) was judged inadequate in our laboratory (Meienhofer *et al.*, 1970a) for the preparation of hepta- γ -L-glutamic acid (176).^{*} The product mixture (see Fig. 11 for a high-voltage paper electrophero-



gram) could not be sufficiently fractionated by several column chromatographic systems to give (176) in homogeneous form. Solution synthesis afforded all protected oligomers (177) $n = 0-5$, in crystalline, analytically pure state, within considerably less time than that spent on the futile attempts to purify the mixture obtained by the solid-phase synthesis. Thus the question which approach would give the best results will largely depend on the nature and properties of the peptide.

In other comparisons Kessler and Iselin (1966) obtained somewhat better yields of Z-Phe-Lys(Boc)-Gly-N₂H₃ by solution synthesis; Takashima *et al.* (1970) found preparations of [4-isoleucine]-deamino-oxytocin to be of equal quality; and the following investigators obtained superior results by the solid-phase method: Jorgensen *et al.* (1969), a purer [Gly¹, Gly²]-Ile⁵-angiotensin II in higher yield; Visser *et al.* (1970a), preparations of [Leu¹³]-S-peptide-(1-13) possessing equal quality; Losse and Klengel (1971), 3-5 times higher yields of homogeneous valinomycin; Ohno *et al.* (1971), higher yields of purer gramicidin S linear protected decapeptide intermediate.

2. Judicious Choice

Which approach should be selected in planning a new synthesis? An investigator with no chemistry training would certainly select the solid-phase procedure. Peptide chemists, on the other hand, tend to adhere to the prov-

^{*} The product was required for a preparation of vitamin B₉ or folic acid conjugate (Godwin *et al.*, 1972).

Table XXIX—Criteria for a Choice Between Solid-Phase Peptide Synthesis (SPS) and Synthesis in Solution (SOL)

Purpose	Preferred method according to number of Amino acid residues					
	3–10	10–20	20–50	50–100	Small protein	Large protein
Intrinsic Activities	SPS	SPS	SPS	SPS	SPS	SPS
Therapeutic Use	SPS	SPS	SPS	SPS	SPS	SPS
Active core	SPS	SPS	SPS	SPS	SPS	—
Identify active principle	SPS	SPS, SOL	SOL	SOL	—	—
Proof of structure	SPS, SOL	SOL	SOL	—	—	—
Conformation study	SPS, SOL	SOL	SOL	—	—	—
Smallest active fragment	SOL	SOL	SOL	—	—	—
ANALOGS						
1. Well-known biology						
a. Highly active	SPS	SPS	SPS	SPS	SPS	SPS
b. Weakly active	(SPS)SOL	SOL	SOL	—	—	—
2. Unknown biology	SOL	SOL	—	—	—	—

en techniques of solution synthesis. It would be more desirable and advisable that the principal consideration in choosing between the two approaches should be the nature of the peptide and of the biological problem. The dilemma facing the investigator is to make a decision concerning the procedure to be selected at a time when the peculiarities of the synthesis are often unknown. There are, however, several pertinent criteria which can be applied in making a project-oriented choice between solid-phase and solution methods. Obviously an important consideration would be the size of the peptide to be prepared. If the purpose of the work is plotted against the size, we obtain a diagram, Table XXIX.

Peptides with over 100 amino acid residues can, at present, only be prepared by solid-phase synthesis. Intrinsic biological activity can ideally be explored by this method. For example, the origin of the lactogenic activity observed in isolated natural human growth hormone was shown by solid-phase synthesis to be intrinsic and not to be due to contaminating lactogenic hormone. Production for therapeutic use, and preparation of an active core of a larger protein, or recognition of an unknown active center by rapid synthesis of many fragments can all be carried out by the solid-phase method.

However, when the homogeneity of the peptide is of prime importance for exploring specific problems such as (a) identification of an unknown biologically active principle found in nature (e.g., thyrotropin releasing hormone), (b) proof of structure of a biologically active peptide, or (c) a con-

formation study, the applicability of the solid-phase method becomes limited to smaller size peptides where one can be reasonably sure that single molecular entities and not mixtures are obtained. To determine the smallest active fragment of a peptide, one definitely does not want to have a contaminating failure peptide in the synthetic product which, by chance, might happen to possess activity. Here only solution synthesis will suffice.

For the synthesis of highly potent analogs of biologically active peptides the solid-phase method can safely be employed. In fact, it is ideally suited for readily providing large number of analogs required for the exploration of structure-activity relationships. However, when activities drop below the 5-10% level, solid-phase procedures become inadequate. An activity of 1% could, for example, be due to 1% of a contaminating failure peptide possessing full activity, or it could be due to 10% of failure peptides having about 10% activity.

Criteria other than the size of the peptide to be prepared should certainly be considered when choosing between solid-phase and solution methods. For instance, the available time and resources or the number of peptides to be prepared are important factors. These criteria might be plotted in a similar fashion as the size. One of the most important aspects which must be carefully considered in making a decision is the purification of the final product as discussed in Section III,F,2.

D. OUTLOOK

The foregoing discussion has attempted to show the momentous stimulation which peptide synthesis received from Merrifield's invention of the solid-phase method. It has also attempted to evaluate implications on the nature and quality of the products in the light of the present imperfections of the method.

The chemical synthesis of proteins has become a declared goal of modern peptide chemistry. Ideally, a true synthesis of a protein must provide assurance for the homogeneity of the product and for its single molecular identity. It is an established tradition in peptide synthesis that uniform products should be prepared. To keep up this tradition, methodological improvements of the solid-phase procedure are required. A great deal of effort is presently being made in this direction, and the results are encouraging.

The solid-phase method is by its concept and implementation ideally suited for the synthesis of large peptides and proteins. However, the presently used experimental conditions and procedures cause considerable limitations in the size of peptides that can be obtained in pure form. It would be highly

desirable if the synthesis of increasingly larger peptides could be preceded or accompanied by appropriate and necessary improvements in the efficiency of the experimental procedure. The goal for the future is the chemical synthesis of proteins for biochemical research. For that purpose, the synthetic products should be obtained in homogeneous form. The task of the peptide chemist is the elaboration of the required methodology. Perhaps, this might be achieved by gradual perfection of the existing efficient procedures.

Acknowledgments

I wish to thank Dr. Eric Atherton, Dr. Ann M. Warner, and Mrs. Mary Middleton for suggestions, criticism, and help in the preparation of this manuscript. I also want to thank Dr. Garland R. Marshall for providing a collection of literature references and Dr. Erich Wunsch for a photograph of crystalline glucagon. Own work described in this article, was supported by NIH grants C-6516 and FR-05526.

References *

- Abramson, F. B., Elliott, D. F., Lindsay, D. G., and Wade, R. (1970). *J. Chem. Soc., C* p. 1042.
- Akiyama, M., Narita, M., and Okawara, M. (1969). *J. Polym. Sci. Part A-1*, **7**, 1299.
- Alarkon, N. L., Lvova, S. D., and Evstigneeva, R. P. (1971). *Khim. Priir. Soedin.* **7**, 474; *Chem. Abstr.* **75**, 141153 (1971).
- Albertson, N. F., and McKay, F. C. (1953). *J. Amer. Chem. Soc.* **75**, 5323.
- Anderer, F. A., Wittmann-Liebold, B., and Wittmann, H. G. (1965). *Z. Naturforsch. B* **20**, 1203.
- Anderson, G. W., and Callahan, F. M. (1958). *J. Amer. Chem. Soc.* **80**, 2902.
- Anderson, G. W., and McGregor, A. C. (1957). *J. Amer. Chem. Soc.* **79**, 6180.
- Anderson, G. W., Zimmerman, J. E., and Callahan, F. (1963). *J. Amer. Chem. Soc.* **85**, 3039.
- Anderson, G. W., Zimmerman, J. E., and Callahan, F. M. (1967). *J. Amer. Chem. Soc.* **89**, 5012.
- Andreatta, R. H., and Scheraga, H. A. (1971). *J. Med. Chem.* **14**, 489.
- Andreatta, R. H., Liem, R. K. H., and Scheraga, H. A. (1971). *Proc. Nat. Acad. Sci. U.S.* **68**, 253.
- Anfinsen, C. B. (1968). *Pure Appl. Chem.* **17**, 461.
- Anfinsen, C. B., and Haber, E. (1961). *J. Biol. Chem.* **236**, 1361.
- Anfinsen, C. B., Ontjes, D., Ohno, M., Corley, L., and Eastlake, A. (1967). *Proc. Nat. Acad. Sci. U. S.* **58**, 1806.
- Anfinsen, C. B., Ontjes, D. A., and Chaiken, I. M. (1971). In "Peptides 1969" (E. Scoffone, ed.), pp. 121-129. North-Holland Publ., Amsterdam.
- Arai, S., Yamashita, M., Kato, H., and Fujimaki, M. (1970). *Agr. Biol. Chem.* **34**, 729.

- Arnold, Z. (1968). *Farm. Pol.* **24**, 371; *Chem. Abstr.* **70**, 58242 (1969).
- Baas, J. M. A., Beyerman, H. C., van de Graaf, B., and de Leer, E. W. B. (1971). In "Peptides 1969" (E. Scoffone, ed.), pp. 173-176. North-Holland Publ., Amsterdam.
- Bailey, J. L. (1967). "Techniques in Protein Chemistry," 2nd ed. Amer. Elsevier, New York.
- Bajusz, S., Medzihradszy, K., Paulay, Z., and Láng, Zs. (1967). *Acta Chim. (Budapest)* **52**, 335.
- Barstow, L. E., Hruby, V. J., Robinson, A. B., Rupley, J. A., Sharp, J. J., and Shimoda, T. (1971). *Fed. Proc., Fed. Amer. Soc. Exp. Biol.* **30**, 1274.
- Bauer, K. (1970). *Z. Naturforsch. B.* **25**, 1125.
- Baugh, C. M., Krumdieck, C. L., Hershman, J. M., and Pittman, J. A., Jr. (1970a) *Endocrinology* **87**, 1015.
- Baugh, C. M., Stevens, J. C., and Krumdieck, C. M. (1970b). *Biochim. Biophys. Acta* **212**, 116.
- Baumert, A., and Jakubke, H. D. (1973). In "Peptides 1971" (H. Nesvadba, ed.). North-Holland Publ. Amsterdam (in press).
- Baxter, J. W. M., Wu, T. C., Manning, M., and Sawyer, W. H. (1969a) *Experientia* **25**, 1127.
- Baxter, J. W. M., Manning, M., and Sawyer, W. H. (1969b). *Biochemistry* **8**, 3592.
- Bayer, E. (1970). In "Peptides Chemistry and Biochemistry" (B. Weinstein and S. Lande, eds.), pp. 99-112. Dekker, New York.
- Bayer, E., and Hagenmaier, H. (1968). *Tetrahedron Lett.* p. 2037.
- Bayer, E., and Koenig, W. A. (1969). *J. Chromatogr. Sci.* **7**, 95.
- Bayer, E., Jung, G., and Hagenmaier, H. (1968a). *Tetrahedron* **24**, 4853.
- Bayer, E., Hagenmaier, H., Jung, G., and König, W. (1968b). In "Peptides 1968" (E. Bricas, ed.), pp. 162-167. North-Holland Publ., Amsterdam.
- Bayer, E., Eckstein, H., Hägele, K., König, W. A., Brüning, W., Hagenmaier, H., and Parr, W. (1970a). *J. Amer. Chem. Soc.* **92**, 1735.
- Bayer, E., Gil-av, E., König, W. A., Nakaparskin, S., Oró, J., and Parr, W. (1970b). *J. Amer. Chem. Soc.* **92**, 1738.
- Bayer, E., Jung, G., Halász, I., and Sebastian, I. (1970c). *Tetrahedron Lett.* p. 4503.
- Bayer, E., Breitmaier, E., Jung, G., and Parr, W. (1971a). *Hoppe-Seyler's Z. Physiol. Chem.* **352**, 759.
- Bayer, E., Hagenmaier, H., Jung, G., Parr, W., Eckstein, H., Hunziker, P., and Sievers, R. E. (1971b). In "Peptides 1969" (E. Scoffone, ed.), pp. 65-73. North-Holland Publ., Amsterdam.
- Beacham, J., Dupuis, G., Finn, F. M., Storey, H. T., Yanaihara, C., Yanaihara, N., and Hofmann, K. (1971). *J. Amer. Chem. Soc.* **93**, 5526.
- Belleau, B., and Malek, G. (1968). *J. Amer. Chem. Soc.* **90**, 1651.
- Ben-Ishai, D., and Berger, A. (1952). *J. Org. Chem.* **17**, 1564.
- Benjamini, E., Shimizu, M., Young, J. D., and Leung, C. Y. (1968a). *Biochemistry* **7**, 1253.
- Benjamini, E., Shimizu, M., Young, J. D., and Leung, C. Y. (1968b). *Biochemistry* **7**, 1261.
- Benjamini, E., Shimizu, M., Young, J. D., and Leung, C. (1969). *Biochemistry* **8**, 2242.
- Bergmann, M., and Zervas, L. (1932). *Ber. Deut. Chem. Ges. B* **65**, 1192.

- Beyerman, H. C. (1972). In "Progress in Peptide Research" (S. Lande, ed.), pp. 25–34. Gordon & Breach, New York.
- Beyerman, H. C., and Hindriks, H. (1971). In "Peptides 1969" (E. Scoffone, ed.), p. 145. North-Holland Publ., Amsterdam.
- Beyerman, H. C., and In't Veld, R. A. (1969). *Rec. Trav. Chim. Pays-Bas* **88**, 1019.
- Beyerman, H. C., and Maassen van den Brink, W. (1963). *Proc. Chem. Soc., London* p. 266.
- Beyerman, H. C., and Maassen van den Brink-Zimmermannová, H. (1968). *Rec. Trav. Chim. Pays-Bas* **87**, 1196.
- Beyerman, H. C., van de Linde, A., and Maassen van den Brink, W., eds. (1967a). "Peptides." North-Holland Publ., Amsterdam.
- Beyerman, H. C., Boers-Boonekamp, C. A. M., van Zoest, W. J., and van den Berg, D. (1967b). In "Peptides" (H. C. Beyerman, A. van de Linde, and W. Maassen van den Brink, eds.), pp. 117–123. North-Holland Publ., Amsterdam.
- Beyerman, H. C., Boers-Boonekamp, C. A. M., and Maassen van den Brink-Zimmermannová, H. (1968a). *Rec. Trav. Chim. Pays-Bas* **87**, 257.
- Beyerman, H. C., Hindriks, H., and de Leer, E. W. B. (1968b). *Chem. Commun.* p. 1668.
- Beyerman, H. C., Hindriks, H., Hirt, J., and de Leer, E. W. B. (1971a). In "Peptides 1969" (E. Scoffone, ed.), pp. 87–91. North-Holland Publ., Amsterdam.
- Beyerman, H. C., Kranenburg, P., and Syrier, J. L. M. (1971b). *Rec. Trav. Chim. Pays-Bas* **90**, 791.
- Beyerman, H. C., van der Kamp, P. R. M., de Leer, E. W. B., Maassen van den Brink, W., Parmentier, J. H., and Westerling, J. (1973a). In "Peptides 1971" (H. Nesvadba, ed.). North-Holland Publ., Amsterdam (in press).
- Beyerman, H. C., Lie, T. S., and van Veldhuizen, C. J. (1973b). In "Peptides 1971" (H. Nesvadba, ed.). North-Holland Publ., Amsterdam (in press).
- Birr, C., and Frodl, R. (1970). *Synthesis* p. 474.
- Birr, C., and Lochinger, W. (1971). *Synthesis* p. 319.
- Birr, C., Flor, F., Fleckenstein, P., Lochinger, W., and Wieland, T. (1973). In "Peptides 1971" (H. Nesvadba, ed.). North-Holland Publ., Amsterdam (in press).
- Blackburn, G. M., Brown, M. J., Harris, M. R., and Shire, D. (1969). *J. Chem. Soc., C* p. 676.
- Blackburn, S. (1970). "Protein Sequence Determination." Dekker, New York.
- Blake, J., and Li, C. H. (1968). *J. Amer. Chem. Soc.* **90**, 5882.
- Blake, J., and Li, C. H. (1971). *Int. J. Protein Res.* **3**, 185.
- Blake, J., Crooks, R. W., and Li, C. H. (1970). *Biochemistry* **9**, 2071.
- Blake, J., Wang, K.-T., and Li, C. H. (1972). *Biochemistry* **11**, 438.
- Blombäck, B., Blombäck, M., Edman, P., and Hessel, B. (1966). *Biochim. Biophys. Acta* **115**, 371.
- Bodanszky, M. (1955). *Nature (London)* **175**, 685.
- Bodanszky, M. (1957). *Acta Chim. (Budapest)* **10**, 335.
- Bodanszky, M. (1970). In "Peptides: Chemistry and Biochemistry" (B. Weinstein and S. Lande, eds.), pp. 1–15. Dekker, New York.
- Bodanszky, M., and Bath, R. J. (1969). *Chem. Commun.* p. 1259.
- Bodanszky, M., and Conklin, L. E. (1967). *Chem. Commun.* p. 773.

- Bodanszky, M., and du Vigneaud, V. (1959). *J. Amer. Chem. Soc.* **81**, 2504.
- Bodanszky, M., and Ondetti, M. A. (1966). "Peptide Synthesis." Wiley, New York.
- Bodanszky, M., and Sheehan, J. T. (1964). *Chem. Ind. (London)* p. 1423.
- Bodanszky, M., and Sheehan, J. T. (1966). *Chem. Ind. (London)* p. 1597.
- Bodanszky, M., Meienhofer, J., and du Vigneaud, V. (1960). *J. Amer. Chem. Soc.* **82**, 3195.
- Bodanszky, M., Ondetti, M. A., Levine, S. D., Narayanan, V. L., von Saltza, M., Sheehan, J. T., Williams, N. J., and Sabo, E. F. (1966). *Chem. Ind. (London)* p. 1757.
- Bodanszky, M., Ondetti, M. A., Levine, S. D., Narayanan, V. L., von Saltza, M., Sheehan, J. T., Williams, N. J., and Sabo, E. F. (1967a). In "Peptides" (H. C. Beyerman, A. van de Linde, and W. Maassen van den Brink, eds.), pp. 242-244. North-Holland Publ., Amsterdam.
- Bodanszky, M., Ondetti, M. A., Levine, S. D., and Williams, N. J. (1967b). *J. Amer. Chem. Soc.* **89**, 6753.
- Boissonnas, R. A. (1951). *Helv. Chim. Acta* **34**, 874.
- Boissonnas, R. A., Guttman, S., and Pless, J. (1966). *Experientia* **22**, 526.
- Bondi, E., Fridkin, M., and Patchornik, A. (1968). *Isr. J. Chem.* **6**, 22.
- Boni, R., Bonora, G. M., Ciceri, L., Gambini, A., Scatturin, A., and Scoffone, E. (1971). *Chim. Ind. (Milan)* **53**, 10 *Chem. Abstr.* **75**, 6284 (1971).
- Bornstein, J., Armstrong, J. McD., Ng, F., Paddle, B. M., and Misconi, L. (1971). *Biochem. Biophys. Res. Commun.* **42**, 252.
- Braunitzer, G., Gehring-Müller, R., Hilschmann, N., Hilse, K., Hombom, G., Rudloff, V., and Wittmann-Liebold, B. (1961). *Hoppe-Seyler's Z. Physiol. Chem.* **325**, 283.
- Braunitzer, G., Hilse, K., Rudloff, V., and Hilschmann, N. (1964). *Advan. Protein Chem.* **19**, 1.
- Brenner, M., and Wehrmüller, J. (1957). *Helv. Chim. Acta* **40**, 2374.
- Brewer, H. B., Jr., and Ronan, R. (1970). *Proc. Nat. Acad. Sci. U. S.* **67**, 1862.
- Bricas, E., ed. (1968). "Peptides 1968." North-Holland Publ., Amsterdam.
- Broadbent, W., Morley, J. S., and Stone, B. E. (1967). *J. Chem. Soc., C* p. 2632.
- Bromer, W. W., Sinn, L. G., Staub, A., and Behrens, O. K. (1956). *J. Amer. Chem. Soc.* **78**, 3858.
- Brown, J. R., and Hartley, B. S. (1966). *Biochem. J.* **101**, 214.
- Bruckner, V., and Medzihradsky, K. (1965). *Acta Chim. (Budapest)* **44**, 5-239.
- Brunfeldt, K. (1969). *Dan. Kemi* **50**, 169.
- Brunfeldt, K., and Halstrøm, J. (1970). *Acta Chem. Scand.* **24**, 3013.
- Brunfeldt, K., Halstrøm, J., and Roepstorff, P. (1968). In "Peptides 1968" (E. Bricas, ed.), pp. 194-196. North-Holland Publ., Amsterdam.
- Brunfeldt, K., Halstrøm, J., and Roepstorff, P. (1969a). *Acta Chem. Scand.* **23**, 2830.
- Brunfeldt, K., Roepstorff, P., and Thomsen, J. (1969b). *Acta Chem. Scand.* **23**, 2906.
- Brunfeldt, K., Roepstorff, P., and Thomsen, J. (1971). In "Peptides 1969" (E. Scoffone, ed.), pp. 148-153. North-Holland Publ., Amsterdam.
- Büchi, H., Steen, K., and Eschenmoser, A. (1963). *Angew. Chem.* **75**, 1176.
- Buka, M., and Zagats, R. (1969). *Latv. PSR Zinat. Akad. Vestis, Khim. Ser.* p. 503; *Chem. Abstr.* **72**, 90857 (1970).

- Bumpus, F. M., Smeby, R. R., and Khairallah, P. A. (1970). In "Peptides: Chemistry and Biochemistry" (B. Weinstein and S. Lande, eds.), pp. 127-150. Dekker, New York.
- Burton, J., Fletcher, G. A., and Young, G. T. (1971). *Chem. Commun.* p. 1057.
- Buzas, A., Egnell, C., and Fréon, P. (1961). *C. R. Acad. Sci.* **252**, 896.
- Camble, R., Garner, R., and Young, G. T. (1968). *Nature (London)* **217**, 247.
- Camble, R., Garner, R., and Young, G. T. (1969). *J. Chem. Soc., C* p. 1911.
- Carpino, L. A. (1957). *J. Amer. Chem. Soc.* **79**, 4427.
- Carpino, L. A., and Han, G. Y. (1970). *J. Amer. Chem. Soc.* **92**, 5748.
- Celis, M. E., Taleisnik, S., and Walter, R. (1971). *Proc. Nat. Acad. Sci. U.S.* **68**, 1428.
- Chaiken, I. M., and Anfinsen, C. B. (1970a). *J. Biol. Chem.* **245**, 2337.
- Chaiken, I. M., and Anfinsen, C. B. (1970b). *J. Biol. Chem.* **245**, 4718.
- Chaiken, I. M., and Anfinsen, C. B. (1971). *J. Biol. Chem.* **246**, 2285.
- Chang, M. M., Leeman, S. E., and Niall, H. D. (1971). *Nature (London), New Biol.* **232**, 86.
- Chaturvedi, N. C., Park, W. K., Smeby, R. R., and Bumpus, F. M. (1970). *J. Med. Chem.* **13**, 177.
- Chillemi, F. (1971). In "Peptides 1969" (E. Scoffone, ed.), pp. 84-86. North-Holland Publ., Amsterdam.
- Chillemi, F., and Merrifield, R. B. (1969). *Biochemistry* **8**, 4344.
- Chillemi, F., and Pecile, A. (1971). *Experientia* **27**, 385.
- Choppin, A. R., and Rogers, J. W. (1948). *J. Amer. Chem. Soc.* **70**, 2967.
- Chou, F. C.-H., Chawla, R. K., Kibler, R. F., and Shapira, R. (1971). *J. Amer. Chem. Soc.* **93**, 267.
- Cramer, F., and Köster, H. (1968). *Angew. Chem., Int. Ed. Engl.* **7**, 473.
- Cuatrecasas, P., and Anfinsen, C. B. (1971a). *Methods Enzymology* **22**, 345.
- Cuatrecasas, P., and Anfinsen, C. B. (1971b). *Annu. Rev. Biochem.* **40**, 775.
- Curtius, T. (1902). *Ber. Deut. Chem. Ges.* **35**, 3226.
- Cusumano, C. L., Taniuchi, H., and Anfinsen, C. B. (1968). *J. Biol. Chem.* **243**, 4769.
- Dane, E., Drees, F., Konrad, P., and Dockner, T. (1962). *Angew. Chem.* **74**, 873.
- Deér, A. (1966). *Angew. Chem., Int. Ed. Engl.* **5**, 1041; *Angew. Chem.* **78**, 1064 (1966).
- Denkewalter, R. G., and Hirschmann, R. (1969). *Amer. Sci.* **57**, 389.
- Denkewalter, R. G., Schwam, H., Strachan, R. G., Beesley, T. E., Veber, D. F., Schoenewaldt, E. F., Barkemeyer, H., Paleveda, W. J., Jr., Jacob, T. A., and Hirschmann, R. (1966). *J. Amer. Chem. Soc.* **88**, 3163.
- Denkewalter, R. G., Veber, D. F., Holly, F. W., and Hirschmann, R. (1969). *J. Amer. Chem. Soc.* **91**, 502.
- Desaullès, P. A., Riniker, B., and Rittel, W. (1969). In "Protein and Polypeptide Hormones" (M. Manganlies, ed.), Int. Congr. Ser. No. 161, p. 489. Excerpta Med. Found., Amsterdam.
- Dorman, L. C. (1969). *Tetrahedron Lett.* p. 2319.
- Dorman, L. C., and Love, J. (1969). *J. Org. Chem.* **34**, 158.
- Dorman, L. C., and Markley, L. D. (1971). *J. Med. Chem.* **14**, 5.
- Dorman, L. C., Markley, L. D., and Mapes, D. A. (1971). *Anal. Biochem.* **39**, 492.

- Dorman, L. C., Nelson, D. A., and Chow, R. C. L. (1972). In "Progress in Peptide Research" (S. Lande, ed.), pp. 65-68. Gordon & Breach, New York.
- Dowling, L. M., and Stark, G. R. (1969). *Biochemistry* **8**, 4728.
- Drenth, J., Jansonius, J. N., Koekoek, R., Swen, H. M., and Wolthers, B. G. (1968). *Nature (London)* **218**, 929.
- Dubos, R. J., and Thompson, R. H. S. (1938). *J. Biol. Chem.* **124**, 501.
- Dunn, F. W., and Stewart, J. M. (1971). *J. Med. Chem.* **14**, 779.
- Dutta, A. S., and Morley, J. S. (1971). *J. Chem. Soc., C* p. 2896.
- Dutta, A. S., and Morley, J. S. (1973). In "Peptides 1971" (H. Nesvadba, ed.). North-Holland Publ., Amsterdam (in press).
- du Vigneaud, V., Ressler, C., Swan, J. M., Roberts, C. W., Katsoyannis, P. G., and Gordon, S. (1953). *J. Amer. Chem. Soc.* **75**, 4879.
- Edmundson, A. B. (1965). *Nature (London)* **205**, 883.
- Elliott, D. F., Moritz, P., and Wade, R. (1973). In "Peptides 1971" (H. Nesvadba, ed.). North-Holland Publ., Amsterdam (in press).
- Elmore, D. T. (1968). "Peptides and Proteins." Cambridge Univ. Press, London and New York.
- Epand, R. F., and Scheraga, H. A. (1968). *Biopolymers* **6**, 1551.
- Epstein, C. J., Goldberger, R. F., Young, D. M., and Anfinsen, C. B. (1962). *Arch. Biochem. Biophys., Suppl.* **1**, 223.
- Erspamer, V., Anastasi, A., Bertaccini, G., and Cei, J. M. (1964). *Experientia* **20**, 489.
- Esko, K., and Karlsson, S. (1970). *Acta Chem. Scand.* **24**, 1415.
- Esko, K., Karlsson, S., and Porath, J. (1968). *Acta Chem. Scand.* **22**, 3342.
- Eylar, E. H., Caccam, J., Jackson, J. J., Westall, F. C., and Robinson, A. B. (1970). *Science* **168**, 1220.
- Eylar, E. H., Brostoff, S., Hashim, G., Caccam, J., and Burnett, P. (1971). *Biol. Chem.* **246**, 5770.
- Fankhauser, P., Brenner, M., Schilling, B., and Fries, P. (1973). In "Peptides 1971" (H. Nesvadba, ed.). North-Holland Publ., Amsterdam (in press).
- Felix, A. M., and Merrifield, R. B. (1970). *J. Amer. Chem. Soc.* **92**, 1385.
- Ferriere, N. (1966). *Sci. Progr., Nature (Paris)*, No. **3372**, 127.
- Finlayson, G. R., and Chrambach, A. (1971). *Anal. Biochem.* **40**, 292.
- Fisher, E. (1907). *Ber. Deut. Chem. Ges.* **40**, 1754.
- Flanigan, E., and Marshall, G. R. (1970). *Tetrahedron Lett.* p. 2403.
- Flanigan, E., and Marshall, G. R. (1972). In "Progress in Peptide Research" (S. Lande, ed.), pp. 7-14. Gordon & Breach, New York.
- Flouret, G. (1970). *J. Med. Chem.* **13**, 843.
- Fontana, A., Veronese, F. M., and Boccu, E. (1971). *Z. Naturforsch. B* **26**, 314.
- Fraefel, W., and du Vigneaud, V. (1970a). *J. Amer. Chem. Soc.* **92**, 1030.
- Fraefel, W., and du Vigneaud, V. (1970b). *J. Amer. Chem. Soc.* **92**, 4426.
- Frankel, M., Ladkany, D., Gilon, C., and Wolman, Y. (1966). *Tetrahedron Lett.* p. 4765.
- Frechet, J. M., and Schnerch, C. (1971). *J. Amer. Chem. Soc.* **93**, 492.
- Freedman, M. H., Cohen, J. S., and Chaiken, I. M. (1971). *Biochem. Biophys. Res. Commun.* **42**, 1148.
- Freer, R. J., and Stewart, J. M. (1972). *Cienc. Cult. (Sao Paulo)* **24**, 539.
- Fridkin, M. (1972). In "Progress in Peptide Research" (S. Lande, ed.), pp. 19-23. Gordon & Breach, New York.

- Fridkin, M., Patchornik, A., and Katchalski, E. (1965a). *J. Amer. Chem. Soc.* **87**, 4646.
- Fridkin, M., Patchornik, A., and Katchalski, E. (1965b). *Isr. J. Chem.* **3**, 69p.
- Fridkin, M., Patchornik, A., and Katchalski, E. (1966a). *J. Amer. Chem. Soc.* **88**, 3164.
- Fridkin, M., Patchornik, A., and Katchalski, E. (1966b). *Isr. J. Chem.* **4**, 59p.
- Fridkin, M., Patchornik, A., and Katchalski, E. (1968). *J. Amer. Chem. Soc.* **90**, 2953.
- Fridkin, M., Patchornik, A., and Katchalski, E. (1971). In "Peptides 1969" (E. Scoffone, ed.), pp. 164-167. North-Holland Publ., Amsterdam.
- Fruton, J. (1949). *Advan. Protein Chem.* **5**, 1.
- Fujii, T., and Sakakibara, S. (1970). *Bull. Chem. Soc. Jap.* **43**, 3954.
- Fujino, M., and Nishimura, O. (1969). *Chem. Pharm. Bull.* **17**, 1937.
- Funatsu, G., Tsugita, A., and Fraenkel-Conrat, H. (1964). *Arch. Biochem. Biophys.* **105**, 25.
- Gabor, G. (1968). *Biopolymers* **6**, 809.
- Garner, R., and Young, G. T. (1969). *Nature (London)* **222**, 177.
- Garner, R., and Young, G. T. (1971). *J. Chem. Soc., C* p. 50.
- Garner, R., Schafer, D. J., Watkins, W. B., and Young, G. T. (1968). In "Peptides 1968" (E. Bricas, ed.), pp. 145-150. North-Holland Publ., Amsterdam.
- Garner, R., Schafer, D. J., and Young, G. T. (1971). In "Peptides 1969" (E. Scoffone, ed.), pp. 102-108. North-Holland Publ., Amsterdam.
- Gawne, G., Kenner, G. W., and Sheppard, R. C. (1969). *J. Amer. Chem. Soc.* **91**, 5669.
- Geiger, R. (1971). *Justus Liebigs Ann. Chem.* **750**, 165.
- Geising, W., and Hörnle, S. (1972). In "Peptides 1971" (H. Nesvadba, ed.). North-Holland Publ., Amsterdam (in press).
- Gish, D. T., Katsoyannis, P. G., Hess, G. P., and Stedman, R. J. (1956). *J. Amer. Chem. Soc.* **78**, 5954.
- Gisin, B. F., and Merrifield, R. B. (1970). In "Peptides: Chemistry and Biochemistry" (B. Weinstein and S. Lande, eds.), pp. 65-77. Dekker, New York.
- Gisin, B. F., Merrifield, R. B., and Tosteson, D. C. (1969). *J. Amer. Chem. Soc.* **91**, 2691.
- Givas, J., Centeno, E. R., Manning, M., and Schon, A. H. (1968). *Immunochemistry* **5**, 314.
- Godwin, H. A., Rosenberg, I. H., Ferenz, C. R., Jacobs, P. M., and Meienhofer, J. (1972). *J. Biol. Chem.* **247**, 2266.
- Goodman, M., and Glaser, C. (1970). In "Peptides: Chemistry and Biochemistry" (B. Weinstein and S. Lande, eds.), pp. 267-335. Dekker, New York.
- Goodman, M., and Kenner, G. W. (1957). *Advan. Protein Chem.* **12**, 465.
- Goodman, M., and McGahren, W. J. (1966). *J. Amer. Chem. Soc.* **88**, 3887.
- Grahl-Nielsen, O., and Tritsch, G. L. (1969). *Biochemistry* **8**, 187.
- Grassmann, W., and Wünsch, E. (1956). *Fortschr. Chem. Org. Naturst.* **13**, 444.
- Greben, A. E., Martinov, V. F., and Titov, M. I. (1968). *Zh. Obshch. Khim.* **38**, 664; *Chem. Abstr.* **69**, 97143 (1968).
- Green, B., and Garson, L. R. (1969). *J. Chem. Soc., C* p. 401.
- Greene, L. J., Stewart, J. M., and Ferreira, S. H. (1970). In "Bradykinin and Related Kinins" (F. Sicuteri, M. Rocha e Silva, and N. Back, eds.), pp. 81-87. Plenum, New York.

- Greenstein, J. P., and Winitz, M. (1961). In "Chemistry of the Amino Acids," Vol. 2, 763–1295. Wiley, New York.
- Gregory, H., Hardy, P. M., Jones, D. S., Kenner, G. W., and Sheppard, R. C. (1964). *Nature (London)* **204**, 931.
- Gross, E. (1971). Personal communication.
- Gross, H., and Bilk, L. (1967). *Angew. Chem.* **79**, 532.
- Gross, H., and Bilk, L. (1968a). In "Peptides 1968" (E. Bricas, ed.), pp. 156–158. North-Holland Publ., Amsterdam.
- Gross, H., and Bilk, L. (1968b). *Tetrahedron* **24**, 6935.
- Gross, H., and Bilk, L. (1969). *Justus Liebigs Ann. Chem.* **725**, 212.
- Gross, H., and Höft, E. (1967). *Angew. Chem., Int. Ed. Engl.* **6**, 335.
- Gut, V., and Rudinger, J. (1968). In "Peptides 1968" (E. Bricas, ed.), pp. 185–188, North-Holland Publ., Amsterdam.
- Gutte, B., and Merrifield, R. B. (1969). *J. Amer. Chem. Soc.* **91**, 501.
- Gutte, B., and Merrifield, R. B. (1970). *Fed. Proc., Fed. Amer. Soc. Exp. Biol.* **29**, 227 (abstr.).
- Gutte, B., and Merrifield, R. B. (1971). *J. Biol. Chem.* **246**, 1922.
- Guttmann, S., and Boissonnas, R. A. (1958). *Helv. Chim. Acta* **41**, 1852.
- Guttmann, S., and Boissonnas, R. A. (1959). *Helv. Chim. Acta* **42**, 1257.
- Guttmann, S., Pless, J., Sandrin, E., Jaquenoud, P.-A., Bossert, H., and Willems, H. (1968). *Helv. Chim. Acta* **51**, 1155.
- Guttmann, S., Pless, J., Huguenin, R. L., Sandrin, E., Bossert, H., and Zehnder, K. (1969). *Helv. Chim. Acta* **52**, 1789.
- Habermann, E., and Jentsch, J. (1967). *Hoppe-Seyler's Z. Physiol. Chem.* **348**, 37.
- Hagenmaier, H. (1970). *Tetrahedron Lett.* p. 283.
- Haglund, H. (1971). *Methods Biochem. Anal.* **19**, 1–104.
- Halász, I., and Sebastian, I. (1969). *Angew. Chem., Int. Ed. Engl.* **8**, 453.
- Halpern, B., and Nitecki, D. E. (1967). *Tetrahedron Lett.* p. 3031.
- Halpern, B., Chew, L., Close, V., and Patton, W. (1968). *Tetrahedron Lett.* p. 5163.
- Halstrøm, J. (1967). *Saetryk Dan. Kemi* **48**, 59.
- Halstrøm, J., and Klostermeyer, H. (1968). *Justus Liebigs Ann. Chem.* **715**, 208.
- Halstrøm, J., Brunfeldt, K., Thomsen, J., and Kovacs, K. (1969). *Acta Chem. Scand.* **23**, 2335.
- Hammerström, K., Lunkenheimer, W., and Zahn, H. (1970). *Makromol. Chem.* **133**, 41.
- Hancock, W. S., Prescott, D. J., Nulty, W. L., Weintraub, J., Vagelos, P. R., and Marshall, G. R. (1971). *J. Amer. Chem. Soc.* **93**, 1799.
- Hartley, B. S., and Kauffman, D. L. (1966). *Biochem. J.* **101**, 229.
- Havran, R. T., Meyers, C., Schwartz, I. L., and Walter, R. (1971a). In "Peptides 1969" (E. Scoffone, ed.), pp. 161–163. North-Holland Publ., Amsterdam.
- Havran, R. T., Schwartz, I. L., Eggena, P., and Walter, R. (1971b). *Intra-Sci. Chem. Reports* **5**, 323.
- Henecka, H. (1952). In "Methoden der organischen Chemie" (E. Müller and O. Bayer, eds.), 4th ed., Vol. 8, p. 521. Houben-Weyl, Stuttgart.
- Hill, R. L. (1968). *Brookhaven Symp. Biol.* **21**, 140.
- Hirs, C. H. W., ed. (1967). "Methods in Enzymology," Vol. 11. Academic Press, New York.
- Hirs, C. H. W., Moore, S., and Stein, W. H. (1960). *J. Biol. Chem.* **235**, 633.
- Hirschmann, R., and Denkwalter, R. G. (1970). *Naturwissenschaften* **57**, 145.

- Hirschmann, R., Nutt, R. F., Veber, D. F., Vitali, R. A., Varga, S. L., Jacob, T. A., Holly, F. W., and Denkwalter, R. G. (1969). *J. Amer. Chem. Soc.* **91**, 507.
- Hiskey, R. G., and Adams, J. B., Jr. (1966). *J. Org. Chem.* **31**, 2178.
- Hjertén, S. (1970). *Methods Biochem. Anal.* **18**, 55-79.
- Hofmann, K. (1973). In "Peptides 1971" (H. Nesvadba, ed.). North-Holland Publ., Amsterdam (in press).
- Hofmann, K., and Katsoyannis, P. G. (1963). In "The Proteins" (H. Neurath, ed.), 2nd ed., Vol. 1, pp. 53-188. Academic Press, New York.
- Hörnle, S. (1967). *Hoppe-Seyler's Z. Physiol. Chem.* **348**, 1355.
- Hörnle, S., and Geising, W. (1971). *Hoppe-Seyler's Z. Physiol. Chem.* **352**, 5.
- Hörnle, S., Weber, U., and Weitzel, G. (1968). *Hoppe-Seyler's Z. Physiol. Chem.* **349**, 1428.
- Horvath, C. G., Preiss, B. A., and Lipsky, S. R. (1967). *Anal. Chem.* **39**, 1422.
- Hruby, V. J., and Ehler, K. W. (1970). *J. Org. Chem.* **35**, 1690.
- Hruby, V. J., Barstow, L. E., and Linhart, T. (1972). *Anal. Chem.* **44**, 343.
- Hutton, J. J., Jr., Marglin, A., Witkop, B., Kurtz, J., Berger, A., and Udenfriend, S. (1968). *Arch. Biochem. Biophys.* **125**, 779.
- Ingwall, R. T., Scheraga, H. A., Lotan, N., Berger, A., and Katchalski, E. (1968). *Biopolymers* **6**, 331.
- Inukai, N., Nakano, K., and Murakami, M. (1967). *Bull. Chem. Soc. Jap.* **40**, 2913.
- Inukai, N., Nakano, K., and Murakami, M. (1968). *Bull. Chem. Soc. Jap.* **41**, 182.
- Iselin, B., Rittel, W., Sieber, P., and Schwyzer, R. (1957). *Helv. Chim. Acta* **40**, 373.
- Ito, Y., and Bowman, R. L. (1971). *Science* **173**, 420.
- Ives, D. A. (1968). *Can. J. Chem.* **46**, 2318.
- Izdebski, J., and Drabarek, S. (1968). *Wiad. Chem.* **22**, 35; *Chem. Abstr.* **69**, 67672 (1968).
- Izumiya, N., Waki, M., Kato, T., Matsuura, S., and Terada, S. (1971). Private communication.
- Izumiya, N., Kato, T., Waki, M., Mitsuyasu, N., Noda, K. Terada, S., and Abe, O. (1972). In "Progress in Peptide Research" (S. Lande, ed.), pp. 69-78. Gordon & Breach, New York.
- Jäger, G., Geiger, R., and Siedel, W. (1968). *Chem. Ber.* **101**, 3537.
- Jakubke, H.-D. (1964). *Chem. Ber.* **97**, 2816.
- Jakubke, H.-D., and Baumert, A. (1973). In "Peptides 1971" (H. Nesvadba, ed.). North-Holland Publ., Amsterdam (in press).
- Jakubke, H.-D., and Jeschkeit, H. (1969). "Aminosäuren, Peptide, Proteine." Akademie-Verlag, Berlin.
- Jakubke, H.-D., and Voigt, A. (1966). *Chem. Ber.* **99**, 2419.
- Jakubke, H.-D., Voigt, A., and Burkhardt, S. (1967). *Chem. Ber.* **100**, 2367.
- Jenkins, S. R., Nutt, R. F., Dewey, R. S., Veber, D. F., Holly, F. W., Paleveda, W. J. Jr., Lanza, T., Jr., Strachan, R. G., Schoenewaldt, E. F., Barkemeyer, H., Dickinson, M. J., Sondey, J., Hirschmann, R., and Walton, E. (1969). *J. Amer. Chem. Soc.* **91**, 505.
- Jeschkeit, H. (1969). *Z. Chem.* **9**, 266.
- Johnson, B. J. (1971). *J. Med. Chem.* **14**, 488.
- Johnson, B. J., and Jacobs, P. M. (1968a). *Chem. Commun.* p. 73.
- Johnson, B. J., and Jacobs, P. M. (1968b). *J. Org. Chem.* **33**, 4524.
- Johnson, B. J., and May, W. P. (1969). *J. Pharm. Sci.* **58**, 1568.
- Johnson, B. J., and Trask, E. G. (1968). *J. Org. Chem.* **33**, 4521.

- Jollès, P., and Jollès, J. (1968). *Helv. Chim. Acta* **51**, 980.
- Jones, J. H., and Young, G. T. (1967). *Chem. Commun.* p. 35.
- Jones, J. H., and Young, G. T. (1968). *J. Chem. Soc., London* p. 436.
- Jorgensen, E. C., and Weinkam, R. J. (1973). In "Peptides 1971" (H. Nesvadba, ed.). North-Holland Publ., Amsterdam (in press).
- Jorgensen, E. C., Windridge, G. C., Patton, W., and Lee, T. C. (1969). *J. Med. Chem.* **12**, 733.
- Jorgensen, E. C., Windridge, G. C., and Lee, T. C. (1970a). *J. Med. Chem.* **13**, 352.
- Jorgensen, E. C., Windridge, G. C., and Lee, T. C. (1970b). *J. Med. Chem.* **13**, 744.
- Jorgensen, E. C., Windridge, G. C., Patton, W., and Lee, T. C. (1970c). In "Peptides: Chemistry and Biochemistry" (B. Weinstein and S. Lande, eds.), pp. 113-125. Dekker, New York.
- Jorgensen, E. S., Windridge, G. C., and Lee, T. C. (1971a). *J. Med. Chem.* **14**, 631.
- Jorgensen, E. C., Rapaka, S. R., Windridge, G. C., and Lee, T. C. (1971b). *J. Med. Chem.* **14**, 899.
- Jorgensen, E. C., Rapaka, S. R., Windridge, G. C., and Lee, T. C. (1971c). *J. Med. Chem.* **14**, 904.
- Jouzier, E. (1969). *Prod. Probl. Pharm.* **24**, 313.
- Jozefczak, C., Bram, G., and Vilkas, M. (1970). *C. R. Acad. Sci.* **271**, 553.
- Kaiser, E., Colescott, R. L., Bossinger, C. D., and Cook, P. I. (1970). *Anal. Biochem.* **34**, 595.
- Karlsson, S., Lindeberg, G., and Ragnarsson, U. (1970a). *Acta Chem. Scand.* **24**, 337.
- Karlsson, S., Lindeberg, G., Porath, J., and Ragnarsson, U. (1970b). *Acta Chem. Scand.* **24**, 1010.
- Kassell, B., and Laskowski, M., Sr. (1965). *Biochem. Biophys. Res. Commun.* **20**, 463.
- Katchalski, E., Sela, M., Silman, H. I., and Berger, A. (1964). In "The Proteins" (H. Neurath, ed.), 2nd ed. Vol. 2, pp. 405-602. Academic Press, New York.
- Kato, I., and Anfinsen, C. B. (1969). *J. Biol. Chem.* **244**, 5849.
- Katsoyannis, P. G., Fukuda, K., Tometsko, A., Suzuki, K., and Tilak, M. (1964). *J. Amer. Chem. Soc.* **86**, 930.
- Keller-Schierlein, W., Mihailovic, M. L., and Prelog, V. (1959). *Helv. Chim. Acta* **42**, 305.
- Kelly, B., and Levy, J. G. (1971). *Biochemistry* **10**, 1763.
- Kemp, D. S. (1973). In "Peptides 1971" (H. Nesvadba, ed.). North-Holland Publ. Amsterdam (in press).
- Kemp, D. S., Wang, S. W., Busby, G., III, and Hugel, G. (1970a). *J. Amer. Chem. Soc.* **92**, 1043.
- Kemp, D. S., Bernstein, Z., and Rebek, J., Jr. (1970b). *J. Amer. Chem. Soc.* **92**, 4756.
- Kenner, G. W., McDermott, J. R., and Sheppard, R. C. (1971). *J. Chem. Soc., D*, p. 636.
- Kessler, W., and Iselin, B. (1966). *Helv. Chim. Acta* **49**, 1330.
- Kettmann, J. R., Jr., Benjamini, E., Michaeli, D., and Leung, D. Y. K. (1967). *Biochem. Biophys. Res. Commun.* **29**, 623.
- Khosla, M. C., Smeby, R. R., and Bumpus, F. M. (1967a). *Biochemistry* **6**, 754.
- Khosla, M. C., Smeby, R. R., and Bumpus, F. M. (1967b). *Science* **156**, 253.
- Khosla, M. C., Chaturvedi, N. C., Smeby, R. R., and Bumpus, F. M. (1968). *Biochemistry* **7**, 3417.

- Khosla, M. C., Smeby, R. R., and Bumpus, F. M. (1972). In "Progress in Peptide Research" (S. Lande, ed.), pp. 41-47. Gordon & Breach, New York.
- Kiryushkin, A. A., Ovchinnikov, Y. A., Kozhevnikova, I. V., and Shemyakin, M. M. (1967). In "Peptides" (H. C. Beyerman, A. van de Linde, and W. Maassen van den Brink, eds.), pp. 100-102. North-Holland Publ., Amsterdam.
- Klieger, E., Schröder, E., and Gibian, H. (1961). *Justus Liebigs Ann. Chem.* **640**, 157.
- Klostermeyer, H. (1968). *Chem. Ber.* **101**, 2823.
- Klostermeyer, H., Halström, J., Kusch, P., Föhles, J., and Lunkenheimer, W. (1967). In "Peptides" (H. C. Beyerman, A. van de Linde, and W. Maassen van den Brink, eds.), pp. 113-116. North-Holland Publ., Amsterdam.
- König, W., and Geiger, R. (1970a). *Chem. Ber.* **103**, 788.
- König, W., and Geiger, R. (1970b). *Chem. Ber.* **103**, 2041.
- Kopple, K. D. (1966). "Peptides and Amino Acids." Benjamin, New York.
- Kopple, K. D., and Renick, R. J. (1958). *J. Org. Chem.* **23**, 1565.
- Kozhukhovskaya, V. M., Lvova, S. D., and Evstigneeva, R. P. (1970). *Khim. Priro. Soedin.* **6**, 599; *Chem. Abstr.* **74**, 126047 (1971).
- Krumdieck, C. L., and Baugh, C. M. (1969). *Biochemistry* **8**, 1568.
- Kung, Y. T., Du, Y. C., Huang, W. T., Chen, C. C., Ke, L. T., Hu, S. C., Jiang, R. Q., Chu, S. Q., Niu, C. I., Hsu, J. Z., Chang, W. C., Chen, L. L., Li, H. S., Wang, Y., Loh, T. P., Chi, A. H., Li, C. H., Shi, P. T., Yieh, Y. H., Tang, K. L., and Hsing, C. Y. (1965). *Sci. Sinica* **14**, 1710.
- Kunitz, M. (1940). *J. Gen. Physiol.* **24**, 15.
- Kupryszewski, G., and Kaczmarek, M. (1961). *Rocz. Chem.* **35**, 931.
- Kusama, T., and Hayatsu, H. (1970). *Chem. Pharm. Bull.* **18**, 319.
- Kusch, P. (1966a). *Kolloid-Z. Z. Polym.* **208**, 138.
- Kusch, P. (1966b). *Angew. Chem.* **78**, 611.
- Lande, S., ed. (1972). "Progress in Peptide Research." Gordon & Breach, New York.
- Laufer, D. A., and Blout, E. R. (1967). *J. Amer. Chem. Soc.* **89**, 1246.
- Laufer, D. A., Chapman, T. M., Marlborough, D. I., Vaidya, V. M., and Blout, E. R. (1968). *J. Amer. Chem. Soc.* **90**, 2696.
- Laursen, R. A. (1966). *J. Amer. Chem. Soc.* **88**, 5344.
- Law, H. D. (1970). "The Organic Chemistry of Peptides." Wiley, New York.
- Lenard, J. (1967). *J. Org. Chem.* **32**, 250.
- Lenard, J. (1969). *Chem. Rev.* **69**, 625.
- Lenard, J., and Robinson, A. B. (1967). *J. Amer. Chem. Soc.* **89**, 181.
- Lenard, J., Schally, A. V., and Hess, G. P. (1964). *Biochem. Biophys. Res. Commun.* **14**, 498.
- Letsinger, R. L., and Kornet, M. J. (1963). *J. Amer. Chem. Soc.* **85**, 3045.
- Letsinger, R. L., Kornet, M. J., Mahadevan, V., and Jerina, D. M. (1964). *J. Amer. Chem. Soc.* **86**, 5163.
- Li, C. H. (1971). Personal communication.
- Li, C. H., and Dixon, J. S. (1971). *Arch. Biochem. Biophys.* **146**, 233.
- Li, C. H., and Hemmami, B. (1972). *J. Med. Chem.* **15**, 697.
- Li, C. H., and Yamashiro, D. (1970). *J. Amer. Chem. Soc.* **92**, 7608.
- Li, C. H., Geschwind, I. I., Levy, A. L., Harris, J. I., Dixon, J. S., Pon, N. G., and Porath, J. O. (1954). *Nature (London)* **173**, 251.
- Li, C. H., Dixon, J. S., and Chung, D. (1958). *J. Amer. Chem. Soc.* **80**, 2587.
- Li, C. H., Dixon, J. S., and Chung, D. (1961). *Biochim. Biophys. Acta* **46**, 324.

- Li, C. H., Barnafi, L., Chrétien, M., and Chung, D. (1965). *Nature (London)* **208**, 1093.
- Li, C. H., Liu, W.-K., and Dixon, J. S. (1966). *J. Amer. Chem. Soc.* **88**, 2050.
- Li, C. H., Dixon, J. S., and Liu, W.-K. (1969). *Arch. Biochem. Biophys.* **133**, 70.
- Lin, M. C., Gutte, B., Moore, S., and Merrifield, R. B. (1970). *J. Biol. Chem.* **245**, 5169.
- Liu, T. J., Stein, W. H., Moore, S., and Elliot, S. D. (1965). *J. Biol. Chem.* **240**, 1143.
- Liu, W.-K., Trzeciak, H., Schüssler, H., and Meienhofer, J. (1971). *Biochemistry* **10**, 2849.
- Lloyd, K., and Young, G. T. (1968). *Chem. Commun.* p. 1400.
- Lloyd, K., and Young, G. T. (1971). *J. Chem. Soc., C* p. 2890.
- Loffet, A. (1967). *Experientia* **23**, 406.
- Loffet, A. (1971). *Hoppe-Seyler's Z. Physiol. Chem.* **352**, 4; *Int. J. Prot. Res.* **3**, 297.
- Loffet, A., and Close, J. (1968). In "Peptides 1968" (E. Bricas, ed.), pp. 189-193. North-Holland Publ., Amsterdam.
- Loffet, A., and Dremier, C. (1971). *Experientia* **27**, 1003.
- Loffet, A., Close, J., and Dremier, C. (1971). In "Peptides 1969" (E. Scoffone, ed.), pp. 92-94. North-Holland Publ., Amsterdam.
- Losse, G., and Klengel, H. (1971). *Tetrahedron* **27**, 1423.
- Losse, G., and Neubert, K. (1968a). *Z. Chem.* **8**, 228.
- Losse, G., and Neubert, K. (1968b). *Z. Chem.* **8**, 387.
- Losse, G., and Neubert, K. (1970a). *Z. Chem.* **10**, 48.
- Losse, G., and Neubert, K. (1970b). *Tetrahedron Lett.* p. 1267.
- Losse, G., Barth, A., and Schatz, K. (1964). *Justus Liebigs Ann. Chem.* **677**, 185.
- Losse, G., Madlung, C., and Lorenz, P. (1968a). *Chem. Ber.* **101**, 1257.
- Losse, G., Grenzer, W., and Neubert, K. (1968b). *Z. Chem.* **8**, 21.
- Lübke, K. (1971). In "Peptides 1969" (E. Scoffone, ed.), pp. 154-156. North-Holland Publ., Amsterdam.
- Lübke, K., and Klostermeyer, H. (1970). *Advan. Enzymol.* **33**, 445.
- Lunkenheimer, W., and Zahn, H. (1970a). *Angew. Makromol. Chem.* **10**, 69.
- Lunkenheimer, W., and Zahn, H. (1970b). *Justus Liebigs Ann. Chem.* **740**, 1.
- Lvova, S. D., Kozhukhovskaya, V. M., Averin, M. N., and Eystigneeva, R. P. (1971). *Khim. Pri. Soedin.* **7**, 466; *Chem. Abstr.* **75**, 152065 (1971).
- McKay, F. C., and Albertson, N. F. (1957). *J. Amer. Chem. Soc.* **79**, 4686.
- MacLaren, J. A., Savige, W. E., and Swan, J. M. (1958). *Aust. J. Chem.* **11**, 345.
- Maher, J. J., Furey, M. E., and Greenberg, L. J. (1971). *Tetrahedron Lett.* p. 27.
- Manecke, G., and Haake, E. (1968). *Naturwissenschaften* **55**, 343.
- Manning, J. M., and Moore, S. (1968). *J. Biol. Chem.* **243**, 5591.
- Manning, M. (1968). *J. Amer. Chem. Soc.* **90**, 1348.
- Manning, M., and Sawyer, W. H. (1970). *Nature (London)* **227**, 715.
- Manning, M., and Sawyer, W. H. (1972). In "Progress in Peptide Research" (S. Lande, ed.), pp. 79-88. Gordon & Breach, New York.
- Manning, M., Wu, T. C., Baxter, J. W. M., and Sawyer, W. H. (1968a). *Experientia* **24**, 659.
- Manning, M., Wu, T.-C., and Baxter, J. W. M. (1968b). *J. Chromatogr.* **38**, 396.
- Manning, M., Coy, E., and Sawyer, W. H. (1970). *Biochemistry* **9**, 3925.
- Mansveld, G. W. H. A., Hindriks, H., and Beyerman, H. C. (1968). In "Peptides 1968" (E. Bricas, ed.), pp. 197-200. North-Holland Publ., Amsterdam.

- Marglin, A. (1969). In "Diabetes" (J. Ostman and R. D. G. Milner, eds.), pp. 395-399. Excerpta Med. Found., Amsterdam.
- Marglin, A. (1971). *Tetrahedron Lett.* p. 3145.
- Marglin, A., and Cushman, S. W. (1967). *Biochem. Biophys. Res. Commun.* **29**, 710.
- Marglin, A., and Merrifield, R. B. (1966). *J. Amer. Chem. Soc.* **88**, 5051.
- Marglin, A., and Merrifield, R. B. (1970). *Annu. Rev. Biochem.* **39**, 841.
- Margoliash, E., Smith, E. L., Kreil, C., and Tuppy, H. (1961). *Nature (London)* **192**, 1121.
- Markley, L. D., and Dorman, L. C. (1970). *Tetrahedron Lett.* p. 1787.
- Marshall, D. L., and Liener, I. E. (1970). *J. Org. Chem.* **35**, 867.
- Marshall, G. R. (1968). *Advan. Exp. Med. Biol.* **2**, 48.
- Marshall, G. R. (1970). In "Peptides: Chemistry and Biochemistry" (B. Weinstein and S. Lande, eds.), pp. 151-162. Dekker, New York.
- Marshall, G. R., and Merrifield, R. B. (1965). *Biochemistry* **4**, 2394.
- Marshall, G. R., and Merrifield, R. B. (1970). In "CRC Handbook of Biochemistry with Selected Data for Molecular Biology" (H. A. Sober, ed.), pp. C145-C162. Chem. Rubber Publ. Co., Cleveland, Ohio.
- Marshall, G. R., Vine, W., and Needleman, P. (1970). *Proc. Nat. Acad. Sci. U.S.* **67**, 1624.
- Marshall, G. R., Eilers, N., and Vine, W. (1972). In "Progress in Peptide Research" (S. Lande, ed.), pp. 15-18. Gordon & Breach, New York.
- Marshall, G. R., Hancock, W. S., Prescott, D. J., Nulty, W. L., Weintraub, J., and Vagelos, P. R. (1973). In "Peptides 1971" (H. Nesvadba, ed.). North-Holland Publ., Amsterdam (in press).
- Mazur, R. H., and Plume, G. (1968). *Experientia* **24**, 661.
- Medzihradsky, K., Kotai, A., Kajtar, M., Szokan, G., and Vajda, T. (1967). *Ann. Univ. Sci. Budapest Rolando Eötvös Nominatae, Sect. Chim.* **9**, 71.
- Mehlis, B., Fischer, W., and Niedrich, H. (1971). In "Peptides 1969" (E. Scoffone, ed.), pp. 146-147. North-Holland Publ., Amsterdam.
- Meienhofer, J. (1962). *Chimia* **16**, 385.
- Meienhofer, J. (1967). *J. Org. Chem.* **32**, 1143.
- Meienhofer, J. (1970). *J. Amer. Chem. Soc.* **92**, 3771.
- Meienhofer, J., ed. (1972). "Chemistry and Biology of Peptides, Proc. 3rd Amer. Peptide Symposium." Ann Arbor Science Publ., Ann Arbor, Michigan.
- Meienhofer, J., and Sano, Y. (1968). *J. Amer. Chem. Soc.* **90**, 2996.
- Meienhofer, J., and Trzeciak, A. (1970). Unpublished data.
- Meienhofer, J., and Trzeciak, A. (1971). *Proc. Nat. Acad. Sci. U.S.* **68**, 1006.
- Meienhofer, J., and Zahn, H. (1960). In "Hoppe-Seyler/Thierfelder Handbuch der physiologisch-und pathologisch-chemischen Analyse" (K. Lang and E. Lehnartz, eds.), 10th ed., Vol. 4, Part 1, pp. 1-115. Springer-Verlag, Berlin and New York.
- Meienhofer, J., Schnabel, E., Bremer, H., Brinkhoff, O., Zabel, R., Sroka, W., Klostermeyer, H., Brandenburg, D., Okuda, T., and Zahn, H. (1963). *Z. Naturforsch. B* **18**, 1120.
- Meienhofer, J., Jacobs, P. M., Godwin, H. A., and Rosenberg, I. H. (1970a). *J. Org. Chem.* **35**, 4137.
- Meienhofer, J., Trzeciak, A., Havran, R. T., and Walter, R. (1970b). *J. Amer. Chem. Soc.* **92**, 7199.

- Meienhofer, J., Trzeciak, A., Douša, T., Hechter, O., Havran, R. T., Schwartz, I. L., and Walter, R. (1971). In "Peptides 1969" (E. Scoffone, ed.), pp. 157-160. North-Holland Publ., Amsterdam.
- Melby, L. R., and Strobach, D. R. (1969a). *J. Org. Chem.* **34**, 421.
- Melby, L. R., and Strobach, D. R. (1969b). *J. Org. Chem.* **34**, 427.
- Meloun, B., Kostka, V., Vanecek, J., Kluh, I., and Sorm, F. (1966). *Collect. Czech. Chem. Commun.* **31**, 321.
- Melrose, G. J. H., Muller, H. K., and Vagg, W. J. (1970). *Nature (London)* **225**, 547.
- Merrifield, R. B. (1962). *Fed. Proc., Fed. Amer. Soc. Exp. Biol.* **21**, 412.
- Merrifield, R. B. (1963). *J. Amer. Chem. Soc.* **85**, 2149.
- Merrifield, R. B. (1964a). *J. Amer. Chem. Soc.* **86**, 304.
- Merrifield, R. B. (1964b). *Biochemistry* **3**, 1385.
- Merrifield, R. B. (1964c). *J. Org. Chem.* **29**, 3100.
- Merrifield, R. B. (1965a). *Science* **150**, 178.
- Merrifield, R. B. (1965b). *Endeavour* **24**, 3.
- Merrifield, R. B. (1966). In "Hypotensive Peptides" (E. G. Erdős, N. Back, and F. Sicuteri, eds.), pp. 1-13. Springer-Verlag, Berlin and New York.
- Merrifield, R. B. (1967). *Recent Progr. Horm. Res.* **23**, 451-482.
- Merrifield, R. B. (1968a). In "CRC Handbook of Biochemistry with Selected Data for Molecular Biology" (H. A. Sober, ed.), pp. C83-C90. Chem. Rubber Publ. Co., Cleveland, Ohio.
- Merrifield, R. B. (1968b). *Sci. Amer.* **218**, 56.
- Merrifield, R. B. (1969a). *Advan. Enzymol.* **32**, 221-296.
- Merrifield, R. B. (1969b). *J. Amer. Med. Ass.* **210**, 1247.
- Merrifield, R. B., and Corigliano, M. A. (1968). *Biochem. Prep.* **12**, 98-103.
- Merrifield, R. B., and Littau, V. (1968). In "Peptides 1968" (E. Bricas, ed.), pp. 179-182. North-Holland Publ. Amsterdam.
- Merrifield, R. B., and Marglin, A. (1967). In "Peptides" (H. C. Beyerman, A. van de Linde, and W. Maassen van den Brink, eds.), pp. 85-90. North-Holland Publ., Amsterdam.
- Merrifield, R. B., and Stewart, J. M. (1965). *Nature (London)* **207**, 522.
- Merrifield, R. B., and Woolley, D. W. (1956). *J. Amer. Chem. Soc.* **78**, 4646.
- Merrifield, R. B., Stewart, J. M., and Jernberg, N. (1966). *Anal. Chem.* **38**, 1905.
- Milne, H. B., and Kilday, W. (1965). *J. Org. Chem.* **30**, 64.
- Milne, H. B., and Most, C. F., Jr. (1968). *J. Org. Chem.* **33**, 169.
- Mitchell, A. R., and Roeske, R. W. (1970). *J. Org. Chem.* **35**, 1171.
- Mitchell, A. R., Gupta, S. K., and Roeske, R. W. (1970). *J. Org. Chem.* **35**, 2877.
- Mitchell, B., and Levy, J. G. (1970). *Biochemistry* **9**, 2762.
- Mitchell, B., Levy, J. G., and Nitz, R. M. (1970). *Biochemistry* **9**, 1839.
- Miura, Y., and Seto, S. (1969). *Bull. Chem. Soc. Jap.* **42**, 3592.
- Miura, Y., Toyama, M., and Seto, S. (1968). *Sci. Rep. Res. Inst., Tohoku Univ., Ser. A* **20**, 41; *Chem. Abstr.* **70**, 47832 (1969).
- Mizoguchi, T., and Woolley, D. W. (1967). *J. Med. Chem.* **10**, 251.
- Mizoguchi, T., Levin, G., Woolley, D. W., and Stewart, J. M. (1968). *J. Org. Chem.* **33**, 903.
- Mizoguchi, T., Shigezane, K., and Takamura, N. (1969). *Chem. Pharm. Bull.* **17**, 411.
- Mizoguchi, T., Shigezane, K., and Takamura, N. (1970). *Chem. Pharm. Bull.* **18**, 1465.

- Morozova, E. A., and Zevail, M. A. (1970a). *Khim. Prir. Soedin.* **6**, 359; *Chem. Abstr.* **73**, 99195 (1970).
- Morozova, E. A., and Zevail, M. A. (1970b). *Zh. Obshch. Khim.* **40**, 1379; *Chem. Abstr.* **74**, 88281 (1971).
- Morozova, E. A., Zevail, M. A., and Zhukova, G. F. (1970). *Zh. Obshch. Khim.* **40**, 1376; *Chem. Abstr.* **74**, 88280 (1971).
- Mukaiyama, T., Matsueda, R., and Maruyama, H. (1970). *Bull. Chem. Soc. Jap.* **43**, 1271.
- Muramatsu, I., and Hagitani, A. (1959). *J. Chem. Soc. Jap. Pure Chem. Sect.* **80**, 1497.
- Mutt, V., and Jorpes, J. E. (1967). *Recent Progr. Horm. Res.* **23**, 483.
- Najjar, V. A., and Merrifield, R. B. (1966). *Biochemistry* **5**, 3765.
- Nanzyo, N., and Sano, S. (1968). *J. Biol. Chem.* **243**, 3431.
- Needleman, S. B., ed. (1970). "Protein Sequence Determination." Springer Publ., New York.
- Neher, R., Riniker, B., Rittel, W., and Zuber, H. (1968). *Helv. Chim. Acta* **51**, 1900.
- Nesvadba, H., ed. (1973). "Peptides 1971." North-Holland Publ. Amsterdam (in press).
- Ney, K. H., and Polzhofer, K. P. (1968). *Tetrahedron* **24**, 6619.
- Niall, H. D. (1971). *Nature (London), New Biol.* **230**, 90.
- Niall, H. D., Keutmann, H. T., Copp, D. H., and Potts, J. T., Jr. (1969). *Proc. Nat. Acad. Sci. U. S.* **64**, 771.
- Niall, H. D., Keutmann, H., Sauer, R., Hogan, M., Dawson, B., Aurbach, G., and Potts, J., Jr. (1970). *Hoppe-Seyler's Z. Physiol. Chem.* **351**, 1586.
- Nitecki, D. E., and Goodman, J. W. (1970). In "Peptides: Chemistry and Biochemistry" (B. Weinstein and S. Lande, eds.), pp. 435-450. Dekker, New York.
- Noda, K., Terada, S., and Izumiya, N. (1970a). *Bull. Chem. Soc. Jap.* **43**, 1883.
- Noda, K., Terada, S., Mitsuyasu, N., Waki, M., Kato, T., and Izumiya, N. (1970b). *Mem. Fac. Sci., Kyushu-Uni., Ser. C.* **7**, 189.
- Noda, K., Terada, S., Mitsuyasu, N., Waki, M., Kato, T., and Izumiya, N. (1971). *Naturwissenschaften* **58**, 147.
- Norris, K., Halstrøm, J., and Brunfeldt, K. (1971). *Acta Chem. Scand.* **25**, 945.
- Ohno, M., and Anfinsen, C. B. (1967). *J. Amer. Chem. Soc.* **89**, 5994.
- Ohno, M., Eastlake, A., Ontjes, D. A., and Anfinsen, C. B. (1969). *J. Amer. Chem. Soc.* **91**, 6842.
- Ohno, M., Kuromizu, K., Ogawa, H., and Izumiya, N. (1971). *J. Amer. Chem. Soc.* **93**, 5251.
- Okuda, T. (1968). *Naturwissenschaften* **55**, 209.
- Okuda, T., and Zahn, H. (1969). *Makromol. Chem.* **121**, 87.
- Okumura, K., Iwasaki, T., and Matsuoka, M. (1971). *Chem. Ind. (London)* p. 929.
- Olsen, R. K. (1970). *J. Heterocycl. Chem.* **7**, 435.
- Omenn, G. S., and Anfinsen, C. B. (1968). *J. Amer. Chem. Soc.* **90**, 6571.
- Ondetti, M. A., Deer, A., Sheehan, J. T., Pluščec, J., and Kocy, O. (1968a). *Biochemistry* **7**, 4069.
- Ondetti, M. A., Narayanan, V. L., von Saltza, M., Sheehan, J. T., Sabo, E. F., and Bodanszky, M. (1968b). *J. Amer. Chem. Soc.* **90**, 4711.
- Ondetti, M. A., Williams, N. J., Sabo, E. F., Pluščec, J., Weaver, E. R., and Kocy, O. (1971). *Biochemistry* **10**, 4033.

- Ontjes, D. A., and Anfinsen, C. B. (1969a). *Proc. Nat. Acad. Sci. U. S.* **64**, 428.
- Ontjes, D. A., and Anfinsen, C. B. (1969b). *J. Biol. Chem.* **244**, 6316.
- Ontjes, D. A., and Anfinsen, C. B. (1970). In "Peptides: Chemistry and Biochemistry" (B. Weinstein and S. Lande, eds.), pp. 79-98. Dekker, New York.
- Oparil, S., Tregear, G. W., Koerner, T., Barnes, B. A., and Haber, E. (1972). *Circ. Res.* **29**, 682.
- Orlowska, A., and Drabarek, S. (1971). *Rocz. Chem.* **45**, 339; *Chem. Abstr.* **75**, 64246 (1971).
- Ostroy, S. E., Lotan, N., Ingwall, R. T., and Scheraga, H. A. (1970). *Biopolymers* **9**, 749.
- Otsuka, H., and Shoji, T. (1967). *Tetrahedron* **23**, 1535.
- Ovchinnikov, Yu. A., Kiryushkin, A. A., and Miroshnikov, A. I. (1965). *Experientia* **21**, 418.
- Ovchinnikov, Yu. A., Kiryushkin, A. A., and Kozhevnikova, I. V. (1968a). *Zh. Obshch. Khim.* **38**, 2631; *Chem. Abstr.* **70**, 78357 (1969).
- Ovchinnikov, Yu. A., Kiryushkin, A. A., and Kozhevnikova, I. V. (1968b). *Zh. Obshch. Khim.* **38**, 2636; *Chem. Abstr.* **70**, 78358 (1969).
- Panetta, C. A., and Casanova, T. G. (1970). *J. Org. Chem.* **35**, 4275.
- Panse, G. T., and Laufer, D. A. (1970). *Tetrahedron Lett.* p. 4181.
- Panse, G. T., and Laufer, D. A. (1972). In "Progress in Peptide Research" (S. Lande, ed.), pp. 3-6. Gordon & Breach, New York.
- Park, W. K., Smeby, R. R., and Bumpus, F. M. (1967). *Biochemistry* **6**, 3458.
- Parr, W., and Grohmann, K. (1971). *Tetrahedron Lett.* p. 2633.
- Paruszewski, R. (1970). *Rocz. Chem.* **44**, 2039; *Chem. Abstr.* **75**, 6405 (1971).
- Paruszewski, R. (1971). *Rocz. Chem.* **45**, 289; *Chem. Abstr.* **75**, 64244 (1971).
- Patchornik, A. (1967). *Advan. Exp. Med. Biol.* **2**, 11.
- Patchornik, A., Fridkin, M., and Katchalski, E. (1967). In "Peptides" (H. C. Beyerman, A. van de Linde, and W. Maassen van den Brink, eds.), pp. 91-99. North-Holland Publ., Amsterdam.
- Patchornik, A., Amit, B., and Woodward, R. B. (1970). *J. Amer. Chem. Soc.* **92**, 6333.
- Paul, R., and Kende, A. S. (1964). *J. Amer. Chem. Soc.* **86**, 741 and 4162.
- Pavars, A. P., Auna, Z. P., and Chipens, G. I. (1971). *Zh. Obshch. Khim.* **41**, 1859. *Chem. Abstr.* **76**, 4152 (1972).
- Pénisson, R., and Zahn, H. (1970). *Makromol. Chem.* **133**, 13.
- Pepper, K. W., Paisley, H. M., and Young, M. A. (1953). *J. Chem. Soc., (London)* p. 4097.
- Pereira, W., Close, V., Patton, W., and Halpern, B. (1969a). *J. Org. Chem.* **34**, 2032.
- Pereira, W., Close, V. A., Jellum, E., Patton, W., and Halpern, B. (1969b). *Aust. J. Chem.* **22**, 1337.
- Perkins, H. R., and Nieto, M. (1970). *Biochem. J.* **116**, 83.
- Peters, T. J., Modha, K., and Drey, C. N. C. (1970). *Biochem. J.* **119**, 20p.
- Pettit, G. R. (1970). "Synthetic Peptides," Vol. 1. Van Nostrand-Reinhold, Princeton, New Jersey.
- Pettit, G. R. (1971). *Ibid.*, Vol. 2.
- Pettit, G. R., and Jones, W. R. (1971). *J. Org. Chem.* **36**, 870.
- Pfiffner, J. J., Calkins, D. G., Bloom, E. S., and O'Dell, B. L. (1946). *J. Amer. Chem. Soc.* **68**, 1392.
- Pietta, P. G., and Marshall, G. R. (1970). *Chem. Commun.* p. 650.

- Pietta, P. G., Cavallo, P. F., and Marshall, G. R. (1973). In "Peptides 1971" (H. Nesvadba, ed.). North-Holland Publ. Amsterdam (in press).
- Pisano, J. J. (1969a). *Science* **163**, 494.
- Pisano, J. J. (1969b). *Science* **165**, 203.
- Polzhofer, K. P. (1969a). *Tetrahedron Lett.* p. 2305.
- Polzhofer, K. P. (1969b). *Tetrahedron* **25**, 4127.
- Polzhofer, K. P. (1971). *Hoppe-Seyler's Z. Physiol. Chem.* **352**, 4.
- Polzhofer, K. P., and Ney, K. H. (1969). *J. Chromatogr.* **43**, 404.
- Polzhofer, K. P., and Ney, K. H. (1970a). *Tetrahedron* **26**, 3221.
- Polzhofer, K. P., and Ney, K. H. (1970b). *Tetrahedron* **26**, 3787.
- Porath, J., Ax  n, R., and Ern  ck, S. (1967). *Nature (London)* **215**, 1491.
- Potapov, V. K., Chekhmakheva, O. G., Shabarova, Z. A., and Prokofev, M. A. (1971). *Dokl. Akad. Nauk. SSSR* **196**, 360; *Chem. Abstr.* **75**, 49481 (1971).
- Potts, J. T., Jr., Tregear, G. W., Keutmann, H. T., Niall, H. D., Sauer, R., Deftos, L. J., Dawson, B. F., Hogan, M. L., and Aurbach, G. D. (1971). *Proc. Nat. Acad. Sci. U. S.* **68**, 63.
- Pourchot, L. M., and Johnson, J. H. (1969). *Org. Prep. Proc.* **1**, 121.
- Ragnarsson, U., Karlsson, S., and Lindeberg, G. (1970a). *Acta Chem. Scand.* **24**, 2821.
- Ragnarsson, U., Lindeberg, G., and Karlsson, S. (1970b). *Acta Chem. Scand.* **24**, 3079.
- Ragnarsson, U., Karlsson, S., and Sandberg, B. (1971). *Acta Chem. Scand.* **25**, 1487.
- Ressler, C. (1956). *J. Amer. Chem. Soc.* **78**, 5956.
- Ressler, C., and du Vigneaud, V. (1954). *J. Amer. Chem. Soc.* **76**, 3107.
- Richards, F. M., and Vithayathil, P. J. (1959). *J. Biol. Chem.* **234**, 1459.
- Richards, F. F., Sloane, R. W., Jr., and Haber, E. (1966). *Fed. Proc., Fed. Amer. Soc. Exp. Biol.* **25**, 653.
- Richards, F. F., Sloane, R. W., Jr., and Haber, E. (1967). *Biochemistry* **6**, 476.
- Riniker, B., Brugger, M., Kamber, B., Sieber, P., and Rittel, W. (1969). *Helv. Chim. Acta* **52**, 1058.
- Riniker, B., Brugger, M., Kamber, B., Rittel, W., and Sieber, P. (1972). In "Progress in Peptide Research" (S. Lande, ed.), pp. 111-120. Gordon & Breach, New York.
- Rittel, W., Brugger, M., Kamber, B., Riniker, R., and Sieber, P. (1968). *Helv. Chim. Acta* **51**, 924.
- Rivaille, P., and Milhaud, G. (1971). *Helv. Chim. Acta* **54**, 355.
- Robinson, A. B., and Kamen, M. D. (1968). In "Structure and Function of Cytochromes" (K. Okumuki, M. D. Kamen, and I. Sekuzu, eds.), pp. 383-387. Univ. Park Press, Baltimore, Maryland.
- Robinson, A. B., McKerrow, J. H., and Cary, P. (1970). *Proc. Nat. Acad. Sci. U. S.* **66**, 753.
- Roepstorff, P., Norris, K., Severinsen, S., and Brunfeldt, K. (1970). *FEBS Lett.* **9**, 235.
- Romanovskaya, I. K., and Chipens, G. I. (1971). *Zh. Obshch. Khim.* **41**, 1856; *Chem. Abstr.* **76**, 4151 (1972).
- Rothe, M., and Dunkel, W. (1967). *J. Polym. Sci., Part B* **5**, 589.
- Rothe, M., and Kunitz, F. W. (1957). *Justus Liebigs Ann. Chem.* **609**, 88.
- Rothe, M., and Schneider, H. J. (1966). *Angew. Chem.* **78**, 390.
- Rothe, M., Schneider, H. J., and Dunkel, W. (1966). *Makromol. Chem* **96**, 290.

- Rothe, M., Dunkel, W., Steffen, K.-D., and Schneider, HJ. (1968). *Angew. Chem.* **80**, 413.
- Rothe, M., Theysohn, R., Steffen, K.-D., Schneider, H.-J., Zamani, M., and Kostrzewa, M. (1969). *Angew. Chem.* **81**, 939.
- Rothe, M., Theysohn, R., Steffen, K.-D., Kostrzewa, M., and Zamani, M. (1971). In "Peptides 1969" (E. Scoffone, ed.), pp. 179–188. North-Holland Publ., Amsterdam.
- Rudinger, J. (1963). *Pure Appl. Chem.* **7**, 335.
- Rudinger, J., and Gut, V. (1967). In "Peptides" (H. C. Beyerman, A. van de Linde, and W. Maassen van den Brink, eds.), pp. 89–90. North-Holland Publ., Amsterdam.
- Ryan, J. W. (1970). *Biochem. J.* **116**, 159.
- Ryle, A. P., Leclerc, J., and Falla, F. (1969). *Biochem. J.* **110**, 4P.
- Rzeszutarska, B., and Wiejak, S. (1968a). *Angew. Chem.* **80**, 364.
- Rzeszutarska, B., and Wiejak, S. (1968b). In "Peptides 1968" (E. Bricas, ed.), pp. 86–89. North-Holland Publ., Amsterdam.
- Rzeszutarska, B., and Wiejak, S. (1968c). *Justus Liebigs Ann. Chem.* **716**, 216.
- Sakai, K., and Anselme, J.-P. (1971). *J. Org. Chem.* **36**, 2387.
- Sakakibara, S. (1971). In "Chemistry and Biochemistry of Amino Acids, Peptides, and Proteins" (B. Weinstein, ed.), pp. 51–85. Dekker, New York.
- Sakakibara, S., and Shimonishi, Y. (1965). *Bull. Chem. Soc. Jap.* **38**, 1412.
- Sakakibara, S., Shimonishi, Y., Okada, M., and Kishida, Y. (1967a). In "Peptides" (H. C. Beyerman, A. van de Linde, and W. Maassen van den Brink, eds.), pp. 44–49. North-Holland Publ., Amsterdam.
- Sakakibara, S., Shimonishi, Y., Kishida, Y., Okada, M., and Sugihara, H. (1967b). *Bull. Chem. Soc. Jap.* **40**, 2164.
- Sakakibara, S., Kishida, Y., Kikuchi, Y., Sakai, R., and Kakiuchi, K. (1968). *Bull. Chem. Soc. Jap.* **41**, 1273.
- Sakakibara, S., Honda, I., Takada, K., Miyosha, M., Ohnishi, T., and Okumura, K. (1969). *Bull. Chem. Soc. Jap.* **42**, 809.
- Sakakibara, S., Fukuda, T., Kishida, Y., and Honda, I. (1970). *Bull. Chem. Soc. Jap.* **43**, 3322.
- Sano, S., and Kurihara, M. (1969). *Hoppe-Seyler's Z. Physiol. Chem.* **350**, 1183.
- Sano, S., Kurihara, M., Nishimura, O., and Yajima, H. (1968). In "Structure and Function of Cytochromes" (K. Okumuki, M. D. Kamen, and I. Sekuzu, eds.), pp. 370–379. University Park Press, Baltimore, Maryland.
- Sano, S., Tokunaga, R., and Kun, K. A. (1971). *Biochim. Biophys. Acta* **244**, 201.
- Sawyer, W. H., and Manning, M. (1971). *J. Endocrinol.* **49**, 151.
- Sawyer, W. H., Wu, T. C., Baxter, J. W. M., and Manning, M. (1969). *Endocrinology* **85**, 385.
- Schafer, D. J., Young, G. T., Elliott, D. F., and Wade, R. (1971). *J. Chem. Soc.*, C p. 46.
- Schaich, E., and Schneider, F. (1971). *Hoppe-Seyler's Z. Physiol. Chem.* **352**, 4.
- Schaich, E., and Schneider, F. (1973). In "Peptides 1971" (H. Nesvadba, ed.). North-Holland Publ., Amsterdam (in press).
- Schleifer, K. H., and Krause, R. M. (1971). *J. Biol. Chem.* **246**, 986.
- Schlossman, S. F., Herman, J., and Yaron, A. (1969). *J. Exp. Med.* **130**, 1031.
- Schnabel, E. (1967). *Justus Liebigs Ann. Chem.* **702**, 188.
- Schnabel, E., Herzog, H., Hoffmann, P., Klauke, E., and Ugi, I. (1968a). *Justus Liebigs Ann. Chem.* **716**, 175.

- Schnabel, E., Herzog, H., Hoffmann, P., Klauke, E., and Ugi, I. (1968b). In "Peptides 1968" (E. Bricas, ed.), pp. 91-97. North-Holland Publ., Amsterdam.
- Schnabel, E., Herzog, H., Hoffmann, P., Klauke, E., and Ugi, I. (1968c). *Angew. Chem.* **80**, 396.
- Schnabel, E., Stoltefuss, J., Offe, H. A., and Klauke, E. (1971a). *Justus Liebigs Ann. Chem.* **743**, 57.
- Schnabel, E., Schmidt, G., and Klauke, E. (1971b). *Justus Liebigs Ann. Chem.* **743**, 69.
- Schnabel, E., Klostermeyer, H., and Berndt, H. (1971c). *Justus Liebigs Ann. Chem.* **749**, 90.
- Schöllmann, G. (1967). *Hoppe-Seyler's Z. Physiol. Chem.* **348**, 1629.
- Schormüller, J., Hans, R., and Belitz, H.-D. (1966). *Z. Lebensm.-Unters. Forsch.* **131**, 65.
- Schreiber, J. (1967). In "Peptides" (H. C. Beyerman, A. van de Linde, and Maassen van den Brink, eds.), p. 107. North-Holland Publ., Amsterdam.
- Schröder, E., and Lübke, K. (1965). "The Peptides," Vol. 1. Academic Press, New York.
- Schröder, E., and Lübke, K. (1966). "The Peptides," Vol. 2. Academic Press, New York.
- Schultze, H. E. (1956). *Z. Elektrochem.* **60**, 262.
- Schwyzer, R., and Sieber, P. (1958). *Helv. Chim. Acta* **41**, 2190.
- Schwyzer, R., and Sieber, P. (1963). *Nature (London)* **199**, 172.
- Schwyzer, R., Feurer, M., and Iselin, B. (1955). *Helv. Chim. Acta* **38**, 83.
- Schwyzer, R., Sieber, P., and Kappeler, H. (1959). *Helv. Chim. Acta* **42**, 2622.
- Scoffone, E., ed. (1971). "Peptides 1969." North-Holland Publ., Amsterdam.
- Scoffone, E., Fontana, A., and Rocchi, R. (1968). *Biochemistry* **7**, 971.
- Scotchler, J., Lozier, R., and Robinson, A. B. (1970). *J. Org. Chem.* **35**, 3151.
- Seiler, N. (1970). *Methods Biochem. Anal.* **18**, 259-337.
- Semkin, E. P., Gafurova, N. D., and Shchukina, L. A. (1967a). *Khim. Prir. Soedin.* **3**, 220; *Chem. Abstr.* **68**, 50037 (1968).
- Semkin, E. P., Smirnova, A. P., and Shchukina, L. A. (1967b). *Zh. Obshch. Khim.* **37**, 1169; *Chem. Abstr.* **68**, 30020 (1968).
- Semkin, E. P., Smirnova, A. P., and Shchukina, L. A. (1968). *Zh. Obshch. Khim.* **38**, 2358; *Chem. Abstr.* **70**, 47838 (1969).
- Shaltiel, S. (1967). *Biochem. Biophys. Res. Commun.* **29**, 178.
- Shaltiel, S., and Fridkin, M. (1970). *Biochemistry* **9**, 5122.
- Shapira, R., Chou, F. C.-H., McKneally, S., Urban, E., and Kibler, R. F. (1971). *Science* **173**, 736.
- Shchukina, L. A., and Sklyarov, L. Y. (1966). *Khim. Prir. Soedin.* **2**, 200; *Chem. Abstr.* **65**, 17042 (1966).
- Shchukina, L. A., Semkin, E. P., and Smirnova, A. P. (1967). *Khim. Prir. Soedin.* **3**, 358; *Chem. Abstr.* **68**, 87567 (1968).
- Shchukina, L. A., Sklyarov, L. Y., and Gorbunov, V. I. (1968). *Khim. Prir. Soedin.* **4**, 120; *Chem. Abstr.* **69**, 87488 (1969).
- Sheehan, J. C., and Grenda, V. J. (1962). *J. Amer. Chem. Soc.* **84**, 2417.
- Sheehan, J. C., and Hess, G. P. (1955). *J. Amer. Chem. Soc.* **77**, 1067.
- Sheehan, J. C., Goodman, M., and Hess, G. P. (1956). *J. Amer. Chem. Soc.* **78**, 1367.
- Sheehan, J. C., Preston, J., and Cruickshank, P. A. (1965). *J. Amer. Chem. Soc.* **87**, 2492.

- Shemyakin, M. M., Ovchinnikov, Yu. A., Kiryushkin, A. A., and Kozhevnikova, I. V. (1965). *Tetrahedron Lett.* p. 2323.
- Shepherd, R. G., Wilson, S. D., Howard, K. S., Bell, P. H., Davis, D. S., Davis, S. B., Eigner, E. A., and Shakespeare, N. E. (1956). *J. Amer. Chem. Soc.* **78**, 5067.
- Sheppard, R. C. (1972). In "Peptides 1971" (H. Nesvadba, ed.). North-Holland Publ., Amsterdam (in press).
- Shimonishi, Y. (1969). *Tampakushitsu, Kakusan, Koso* **13**, 651; *Chem. Abstr.* **69**, 97108 (1968).
- Sieber, P., and Iselin, B. (1968a). *Helv. Chim. Acta* **51**, 614.
- Sieber, P., and Iselin, B. (1968b). *Helv. Chim. Acta* **51**, 622.
- Sieber, P., Brugger, M., Kamber, B., Riniker, B., and Rittel, W. (1968). *Helv. Chim. Acta* **51**, 2057.
- Sieber, P., Riniker, B., Brugger, M., Kamber, B., and Rittel, W. (1970). *Helv. Chim. Acta* **53**, 2135.
- Sipos, F., and Gaston, D. W. (1971). *Synthesis* p. 321.
- Skeggs, L. T., Lentz, K. E., Kahn, J. R., Dorer, F. E., and Levine, M. (1969). *Circ. Res.* **25**, 451.
- Sklyarov, L. Yu., and Shashkova, I. V. (1969). *Zh. Obshch. Khim.* **39**, 2778; *Chem. Abstr.* **72**, 121903 (1970).
- Sklyarov, L. Yu., Gorbunov, V. I., and Shchukina, L. A. (1966). *Zh. Obshch. Khim.* **36**, 2220; *Chem. Abstr.* **66**, 115945 (1967).
- Smirnov, V. D., Khangulov, G. A., Shabarova, Z. A., and Prokofev, M. A. (1969). *Vestn. Mosk. Univ.* **24**, 118; *Chem. Abstr.* **72**, 55864 (1970).
- Smith, R. L., and Dunn, F. W. (1966). *Fed. Proc., Fed. Amer. Soc. Exp. Biol.* **25**, 801.
- Smith, R. L., and Dunn, F. W. (1970). *J. Biol. Chem.* **245**, 2962.
- Smith, R. L., Archer, E. G., and Dunn, F. W. (1970). *J. Biol. Chem.* **245**, 2967.
- Smyth, D. G., Stein, W. H., and Moore, S. (1962). *J. Biol. Chem.* **237**, 1845.
- Smyth, D. G., Stein, W. H., and Moore, S. (1963). *J. Biol. Chem.* **238**, 227.
- Southard, G. L. (1971). In "Peptides 1969" (E. Scoffone, ed.), pp. 168-170. North-Holland Publ., Amsterdam.
- Southard, G. L., Brooke, G. S., and Pettee, J. M. (1969). *Tetrahedron Lett.* p. 3505.
- Southard, G. L., Brooke, G. S., and Pettee, J. M. (1971a). *Tetrahedron* **27**, 1359.
- Southard, G. L., Brooke, G. S., and Pettee, J. M. (1971b). *Tetrahedron* **27**, 2701.
- Southard, G. L., Brooke, G. S., and Pettee, J. M. (1971c). In "Peptides 1969" (E. Scoffone, ed.), pp. 95-101. North-Holland Publ., Amsterdam.
- Spitler, L., Benjamini, E., Young, J. D., Kaplan, H., and Fudenberg, H. H. (1970). *J. Exp. Med.* **131**, 133.
- Staab, H. A. (1962). *Angew. Chem., Int. Ed. Engl.* **1**, 351.
- Staab, H. A., and Wendel, K. (1963). *Chem. Ber.* **96**, 3374.
- Steinman, G. (1967a). *Arch. Biochem. Biophys.* **119**, 76.
- Steinman, G. (1967b). *Experientia* **23**, 177.
- Stewart, F. H. C. (1967). *Aust. J. Chem.* **20**, 2243.
- Stewart, J. M. (1968). *Fed. Proc., Fed. Amer. Soc. Exp. Biol.* **27**, 534.
- Stewart, J. M., and Woolley, D. W. (1965a). *Nature (London)* **206**, 619.
- Stewart, J. M., and Woolley, D. W. (1965b). *Nature (London)* **207**, 1160.

- Stewart, J. M., and Woolley, D. W. (1966). In "Hypotensive Peptides" (E. G. Erdös, N. Back, and F. Sicuteri, eds.), pp. 23-31. Springer-Verlag, Berlin and New York.
- Stewart, J. M., and Young, J. D. (1969). "Solid Phase Peptide Synthesis." Freeman, San Francisco, California.
- Stewart, J. M., Young, J. D., Benjamini, E., Shimizu, M., and Leung, C. Y. (1966). *Biochemistry* **5**, 3396.
- Stewart, J. M., Mizoguchi, T., and Woolley, D. W. (1967). *153rd Amer. Chem. Soc. Meet., Miami, 1967* Abstr. Pap. 0-206.
- Stewart, J. M., Knight, M., Paiva, A. C. M., and Paiva, T. (1972). In "Progress in Peptide Research" (S. Lande, ed.), pp. 59-64. Gordon & Breach, New York.
- Strachan, R. G., Paleveda, W. J., Jr., Nutt, R. F., Vitali, R. A., Veber, D. F., Dickinson, M. J., Garsky, V., Deak, J. E., Walton, E., Jenkins, S. R., Holly, F. W., and Hirschmann, R. (1969). *J. Amer. Chem. Soc.* **91**, 503.
- Stryer, L., and Haugland, R. (1967). *Proc. Nat. Acad. Sci. U.S.* **58**, 719.
- Sugiyama, H., Miura, Y., and Seto, S. (1968). *Yuki Gosei Kagaku Kyokai Shi* **26**, 1010; *Chem. Abstr.* **70**, 58264 (1969).
- Takahashi, K. (1965). *J. Biol. Chem.* **240**, 4117.
- Takashima, H., and du Vigneaud, V. (1970). *J. Amer. Chem. Soc.* **92**, 2501.
- Takashima, H., du Vigneaud, V., and Merrifield, R. B. (1968). *J. Amer. Chem. Soc.* **90**, 1323.
- Takashima, H., Fraefel, W., and du Vigneaud, V. (1969). *J. Amer. Chem. Soc.* **91**, 6182.
- Takashima, H., Hruby, V. J., and du Vigneaud, V. (1970). *J. Amer. Chem. Soc.* **92**, 677.
- Tanaka, M., Nakashima, T., Benson, A., Mower, H., and Yasunobu, K. T. (1966). *Biochemistry* **5**, 1666.
- Taniuchi, H., Anfinsen, C. B., and Sodja, A. (1967). *J. Biol. Chem.* **242**, 4752.
- Taniuchi, H., Cusumano, C. L., Anfinsen, C. B., and Cone, J. L. (1968). *J. Biol. Chem.* **243**, 4775.
- Tarbell, D. S., and Insalaco, M. A. (1967). *Proc. Nat. Acad. Sci. U.S.* **57**, 233.
- Taschner, E., Rzesotarska, B., and Kuziel, A. (1965). *Acta Chim. Budapest.* **44**, 11.
- Tesser, G. I., and Ellenbroek, B. W. J. (1967). In "Peptides" (H. C. Beyerman, A. van de Linde, and W. Maassen van den Brink, eds.), p. 124. North-Holland Publ., Amsterdam.
- Thampi, N. S., and Huggins, C. G. (1968). *Fed. Proc., Fed. Amer. Soc. Exp. Biol.* **27**, 533.
- Thampi, N. S., Schoellmann, G., Hurst, M. W., and Huggins, C. G. (1968). *Life Sci.* **7**, 641.
- Thomas, A. B., and Rochow, E. G. (1957). *J. Amer. Chem. Soc.* **79**, 1843.
- Thomas, J. O. (1967). *Tetrahedron Lett.* p. 335.
- Thompson, K. E., and Levy, J. G. (1970). *Biochemistry* **9**, 3463.
- Tilak, M. A. (1968). *Tetrahedron Lett.* p. 6323.
- Tilak, M. A. (1970). *Tetrahedron Lett.* p. 849.
- Tilak, M. A., and Hollinden, C. S. (1968a). *Tetrahedron Lett.* p. 1297.
- Tilak, M. A., and Hollinden, C. S. (1968b). In "Peptides 1968" (E. Bricas, ed.), pp. 173-178. North-Holland Publ., Amsterdam.
- Tilak, M. A., and Hollinden, C. S. (1971). *Org. Prep. Proc.* **3**, 183.

- Tometsko, A. M., Tischio, J., and Garden, J., II. (1970). *J. Pharm. Sci.* **59**, 1655.
- Tometsko, A. M., Garden, J., II, and Tischio, J. (1971). *Rev. Sci. Instrum.* **42**, 331.
- Tregear, G. W., Catt, K., and Niall, H. D. (1967). *Proc. Roy. Austr. Chem. Inst.*, p. 345.
- Tregear, G. W., Niall, H. D., Potts, J. T., Jr., Leeman, S. E., and Chang, M. M. (1971). *Nature (London) New Biol.* **232**, 87.
- Tripp, S. L. (1968). *Diss. Abstr. B (London)*, **28**, 3661.
- Tritsch, G. L., and Grahl-Nielsen, O. (1969). *Biochemistry* **8**, 1816.
- Truce, W. E., Campbell, R. W., and Norell, J. R. (1964). *J. Amer. Chem. Soc.* **86**, 288.
- Vanaman, T. C., Wakil, S. J., and Hill, R. L. (1968). *J. Biol. Chem.* **243**, 6420.
- Vane, J. R. (1971). *Nature (London)* **230**, 382.
- van Mourik, A. S., Harryvan, E., and Arens, J. F. (1965). *Rec. Trav. Chim. Pays-Bas* **84**, 1344.
- Varsel, C., Nuwayser, E. S., and Richard, A. J. (1970). *Biochim. Biophys. Acta* **200**, 417.
- Vaughan, J. R., Jr., (1951). *J. Amer. Chem. Soc.* **73**, 3547.
- Vdovina, R. G., Gracheva, A. K., Poznyak, M. G., and Shvachkin, Yu. P. (1971). *Zh. Obshch. Khim.* **41**, 239; *Chem. Abstr.* **75**, 20975 (1971).
- Veber, D. F., Milkowski, J. D., Denkwalter, R. G., and Hirschmann, R. (1968). *Tetrahedron Lett.* p. 3057.
- Veber, D. F., Varga, S. L., Milkowski, D., Joshua, H., Conn, J. B., Hirschmann, R., and Denkwalter, R. G. (1969). *J. Amer. Chem. Soc.* **91**, 506.
- Vesa, V. S. (1968). *Usp. Khim.* **37**, 246; *Chem. Abstr.* **69**, 27748 (1969).
- Vesa, V. S., and Dirvianskyte, N. (1968). *Liet. TSR Mokslu Akad. Darb, Ser. B.* p. 59; *Chem. Abstr.* **70**, 20321 (1969).
- Viehe, H. G., Fuks, R., and Reinstein, M. (1964). *Angew. Chem.* **76**, 571.
- Visser, S., and Kerling, K. E. T. (1970). *Rec. Trav. Chim. Pays-Bas* **89**, 880.
- Visser, S., Roeloffs, J., Kerling, K. E. T., and Havinga, E. (1968). *Rec. Trav. Chim. Pays-Bas* **87**, 559.
- Visser, S., Raap, J., Kerling, K. E. T., and Havinga, E. (1970a). *Rec. Trav. Chim. Pays-Bas* **89**, 865.
- Visser, S., Kerling, K. E. T., and Havinga, E. (1970b). *Rec. Trav. Chim. Pays-Bas* **89**, 876.
- Vlasov, G. P., and Bilibin, A. Yu. (1969). *Izv. Akad. Nauk SSSR, Ser. Khim.* p. 1400; *Chem. Abstr* **71**, 81710 (1969).
- Vogler, K., and Studer, R. O. (1966). *Experientia* **22**, 345.
- Walter, R., and Havran, R. T. (1971). *Experientia* **27**, 645.
- Wang, S.-S., and Merrifield, R. B. (1969a). *J. Amer. Chem. Soc.* **91**, 6488.
- Wang, S.-S., and Merrifield, R. B. (1969b). *Int. J. Protein Res.* **1**, 235.
- Wang, S.-S., and Merrifield, R. B. (1971). In "Peptides 1969" (E. Scoffone, ed.), pp. 74-83. North-Holland Publ., Amsterdam.
- Weber, U. (1969). *Hoppe-Seyler's Z. Physiol. Chem.* **350**, 1421.
- Weber, U., and Weitzel, G. (1968). *Hoppe-Seyler's Z. Physiol. Chem.* **349**, 1431.
- Weber, U., Hörnle, S., Grieser, G., Herzog, K.-H., and Weitzel, G. (1967). *Hoppe-Seyler's Z. Physiol. Chem.* **348**, 1715.
- Weber, U., Hörnle, S., Köhler, P., Nagelschneider, G., Eisele, K., and Weitzel, G. (1968). *Hoppe-Seyler's Z. Physiol. Chem.* **349**, 512.
- Weber, U., Herzog, K.-H., Grossmann, H., Hörnle, S., and Weitzel, G. (1969). *Hoppe-Seyler's Z. Physiol. Chem.* **350**, 1425.

- Weber, U., Herzog, K.-H., Grossmann, H., Hartter, P., and Weitzel, G. (1971). *Hoppe-Seyler's Z. Physiol. Chem.* **352**, 419.
- Weinstein, B., and Lande, S. (1968). *Science* **162**, 1511.
- Weinstein, B., and Lande, S., eds. (1970). "Peptides: Chemistry and Biochemistry." Dekker, New York.
- Weitzel, G., Weber, U., Hörnle, S., and Schneider, F. (1968a). In "Peptides 1968" (E. Bricas, ed.), pp. 171-172. North-Holland Publ., Amsterdam.
- Weitzel, G., Weber, U., Hörnle, S., and Schneider, F. (1968b). In "Peptides 1968" (E. Bricas, ed.), pp. 222-227. North-Holland Publ., Amsterdam.
- Weitzel, G., Eisele, K., Zollner, H., and Weber, U. (1969). *Hoppe-Seyler's Z. Physiol. Chem.* **350**, 1480.
- Weitzel, G., Weber, U., Eisele, K., Zollner, H., and Martin, J. (1970). *Hoppe-Seyler's Z. Physiol. Chem.* **351**, 263.
- Westall, F. C., and Robinson, A. B. (1970). *J. Org. Chem.* **35**, 2842.
- Westall, F. C., Robinson, A. B., Caccam, J., Jackson, J., and Eylar, E. H. (1971). *Nature (London)* **229**, 22.
- Westley, J. W., and Halpern, B. (1968). In "Gas Chromatography 1968" (C. L. A. Halbourne, ed.), pp. 119-128. Institute of Petroleum, London.
- Weygand, F. (1968). In "Peptides 1968" (E. Bricas, ed.), pp. 183-184. North-Holland Publ., Amsterdam.
- Weygand, F., and Obermeier, R. (1968). *Z. Naturforsch. B* **23**, 1390.
- Weygand, F., and Ragnarsson, U. (1966). *Z. Naturforsch. B* **21**, 1141.
- Weygand, F., and Steglich, W. (1959). *Z. Naturforsch. B* **14**, 472.
- Weygand, F., and Zumach, G. (1962). *Z. Naturforsch. B* **17**, 807.
- Weygand, F., Hoffmann, D., and Wunsch, E. (1966). *Z. Naturforsch. B* **21**, 426.
- Weygand, F., Huber, P., and Weiss, K. (1967). *Z. Naturforsch. B* **22**, 1084.
- Weygand, F., Steglich, W., Fraunberger, F., Pietta, P., and Schmid, J. (1968a). *Chem. Ber.* **101**, 923.
- Weygand, F., Steglich, W., and Chytil, N. (1968b). *Z. Naturforsch. B* **23**, 1391.
- White, F. H., Jr. (1961). *J. Biol. Chem.* **236**, 1353.
- Wieland, T. (1968). *Science* **159**, 946.
- Wieland, T., and Bernhard, H. (1951). *Justus Liebigs Ann. Chem.* **572**, 190.
- Wieland, T., and Birr, C. (1966). *Angew. Chem.* **78**, 303; *Angew. Chem., Int. Ed. Engl.* **5**, 310 (1966).
- Wieland, T., and Birr, C. (1967a). *Chimia* **21**, 581.
- Wieland, T., and Birr, C. (1967b). In "Peptides" (H. C. Beyerman, A. van de Linde, and W. Maassen van den Brink, eds.), pp. 103-106. North-Holland Publ., Amsterdam.
- Wieland, T., and Gebert, U. (1966). *Justus Liebigs Ann. Chem.* **700**, 157.
- Wieland, T., and Heinke, B. (1956). *Justus Liebigs Ann. Chem.* **599**, 70.
- Wieland, T., and Hennig, H. J. (1960). *Chem. Ber.* **93**, 1236.
- Wieland, T., and Mohr, H. (1956). *Justus Liebigs Ann. Chem.* **599**, 222.
- Wieland, T., and Racky, W. (1968). *Chimia* **22**, 375.
- Wieland, T., Lüben, G., Ottenheym, H., Faesel, J., de Vries, J. X., Konz, W., Prox, A., and Schmid, J. (1968). *Angew. Chem.* **80**, 209; *Angew. Chem., Int. Ed. Engl.* **7**, 204 (1968).
- Wieland, T., Birr, C., and Flor, F. (1969a). *Justus Liebigs Ann. Chem.* **727**, 130.
- Wieland, T., Birr, C., and Wissenbach, H. (1969b). *Angew. Chem.* **81**, 782; *Angew. Chem., Int. Ed. Engl.* **8**, 764 (1969).

- Wieland, T., Faesel, J., and Konz, W. (1969c). *Justus Liebigs Ann. Chem.* **722**, 197.
- Wieland, T., Lewalter, J., and Birr, C. (1970). *Justus Liebigs Ann. Chem.* **740**, 31.
- Wieland, T., von Dungen, A., and Birr, C. (1971a). *FEBS Lett.* **14**, 299.
- Wieland, T., Birr, C., and Flor, F. (1971b). *Angew. Chem.* **83**, 333; *Angew. Chem. Int. Ed. Engl.* **10**, 336 (1971).
- Wieland, T., Birr, C., and von Dungen, A. (1971c). *Justus Liebigs Ann. Chem.* **747**, 207.
- Wildi, B. S., and Johnson, J. H. (1968). *Abstr., 155th Amer. Chem. Soc. Meet., San Francisco, 1968* Abstr. Pap. A8.
- Windridge, G. C., and Jorgensen, E. C. (1971a). In "Intra-Science Chem. Reports" **5**, 375.
- Windridge, G. C., and Jorgensen, E. C. (1971b). *J. Amer. Chem. Soc.* **93**, 6318.
- Wissmann, H., and Geiger, R. (1970). *Angew. Chem.* **82**, 937; *Angew. Chem. Int. Ed. Engl.* **9**, 908 (1970).
- Wolman, Y., Gallop, P. M., and Patchornik, A. (1961). *J. Amer. Chem. Soc.* **83**, 1263.
- Wolman, Y., Gallop, P. M., Patchornik, A., and Berger, A. (1962). *J. Amer. Chem. Soc.* **84**, 1889.
- Wolman, Y., Kivity, S., and Frankel, M. (1967). *Chem. Commun.* p. 629.
- Woodward, R. B., and Olofson, R. A. (1961). *J. Amer. Chem. Soc.* **83**, 1007.
- Woolley, D. W. (1966). *J. Amer. Chem. Soc.* **88**, 2309.
- Wünsch, E. (1967). *Z. Naturforsch. B* **22**, 1269.
- Wünsch, E. (1971). *Angew. Chem.* **83**, 773.
- Wünsch, E., and Drees, F. (1966). *Chem. Ber.* **99**, 110.
- Wünsch, E., and Wendlberger, G. (1967). *Chem. Ber.* **100**, 820.
- Wünsch, E., and Wendlberger, G. (1968). *Chem. Ber.* **101**, 3659.
- Wünsch, E., Fontana, A., and Drees, F. (1967). *Z. Naturforsch. B* **22**, 607.
- Wünsch, E., Zwick, A., and Fontana, A. (1968a). *Chem. Ber.* **101**, 326.
- Wünsch, E., Zwick, A., and Jaeger, E. (1968b). *Chem. Ber.* **101**, 336.
- Wünsch, E., Jaeger, E., and Scharf, R. (1968c). *Chem. Ber.* **101**, 3664.
- Wünsch, E., Wendlberger, G., Jaeger, E., and Scharf, R. (1968d). In "Peptides 1968" (E. Bricas, ed.), pp. 229-236. North-Holland Publ., Amsterdam.
- Yajima, H., and Kawatani, H. (1968). *Chem. Pharm. Bull.* **16**, 182.
- Yajima, H., and Kubo, K. (1965a). *Chem. Pharm. Bull.* **13**, 759.
- Yajima, H., and Kubo, K. (1965b). *Biochim. Biophys. Acta* **97**, 596.
- Yajima, H., Kawatani, H., and Kiso, Y. (1970a). *Chem. Pharm. Bull.* **18**, 850.
- Yajima, H., Kawatani, H., and Watanabe, H. (1970b). *Chem. Pharm. Bull.* **18**, 1279.
- Yajima, H., Kawatani, H., and Watanabe, H. (1970c). *Chem. Pharm. Bull.* **18**, 1333.
- Yamashiro, D., Blake, J., and Li, C. H. (1972). *J. Amer. Chem. Soc.* **94**, 2855.
- Yanaihara, N., Yanaihara, C., Dupuis, G., Beacham, J., Camble, R., and Hofmann, K. (1969). *J. Amer. Chem. Soc.* **91**, 2184.
- Yanaihara, N., Hashimoto, T., Yanaihara, C., and Sakura, N. (1970). *Chem. Pharm. Bull.* **18**, 417.
- Yaoi, Y., Titani, K., and Narita, K. (1966). *J. Biochem. (Tokyo)* **59**, 247.
- Yaron, A., and Schlossman, S. F. (1966). *Isr. J. Chem.* **4**, 69p.
- Yaron, A., and Schlossman, S. F. (1968). *Biochemistry* **7**, 2673.

- Young, G. T., ed. (1963). "Peptides." Pergamon, Oxford.
- Young, G. T., ed. (1969). "Amino-acids, Peptides, and Proteins—A Specialist Periodical Report," Vol. 1. Chemical Society, London.
- Young, G. T., ed. (1970). "Amino-acids, Peptides, and Proteins—A Specialist Periodical Report," Vol. 2. Chemical Society, London.
- Young, G. T., ed. (1971). "Amino-acids, Peptides, and Proteins—A Specialist Periodical Report," Vol. 3. Chemical Society, London.
- Young, J. D., Benjamini, E., Stewart, J. M., and Leung, C. Y. (1967). *Biochemistry* **6**, 1455.
- Young, J. D., Leung, C. Y., and Rombauts, W. A. (1968a). *Biochemistry* **7**, 2475.
- Young, J. D., Benjamini, E., and Leung, C. Y. (1968b). *Biochemistry* **7**, 3113.
- Young, J. D., Voelter, W., Shimizu, M., Leung, C. Y., Peterson, W. J., and Benjamini, E. (1972). In "Progress in Peptide Research" (S. Lande, ed.), pp. 35–39. Gordon & Breach, New York.
- Zahn, H., and Diehl, J. F. (1957a). *Z. Naturforsch. B* **12**, 85.
- Zahn, H., and Diehl, J. F. (1957b). *Angew. Chem.* **69**, 135.
- Zahn, H., Okuda, T., and Shimonishi, Y. (1967a). *Angew. Chem.* **79**, 424; *Angew. Chem. Int. Ed. Engl.* **6**, 460 (1967).
- Zahn, H., Okuda, T., and Shimonishi, Y. (1967b). In "Peptides" (H. C. Beyerman, A. van de Linde, and W. Maassen van den Brink, eds.), pp. 108–112. North-Holland Publ., Amsterdam.
- Zahn, H., Berndt, H., Fehrenbach, P., von Wachtendonk, D., and Klostermeyer, H. (1973). In "Peptides 1971" (H. Nesvadba, ed.). North-Holland Publ., Amsterdam (in press).
- Zeiger, A., and Anfinsen, C. B. (1972). In "Progress in Peptide Research" (S. Lande, ed.), pp. 307–316. Gordon & Breach, New York.
- Zervas, L., ed. (1966). "Peptides." Pergamon, Oxford.
- Zervas, L., and Hamalidis, C. (1965). *J. Amer. Chem. Soc.* **87**, 99.
- Zervas, L., Borovas, D., and Gazis, E. (1963). *J. Amer. Chem. Soc.* **85**, 3660.
- Zevail, M. A., and Morozova, E. A. (1970). *Zh. Obshch. Khim.* **40**, 2760; *Chem. Abstr.* **75**, 36635 (1971).
- Zevail, M. A., and Morozova, E. A. (1971). *Vestn. Mosk. Univ., Khim.* **12**, 246; *Chem. Abstr.* **75**, 36623 (1971).
- Zhukova, G. F., Ravdel, G. A., and Shchukina, L. A. (1970). *Zh. Obshch. Khim.* **40**, 2753; *Chem. Abstr.* **74**, 76647 (1971).
- Zuber, H. (1960). *Chimia* **14**, 405.