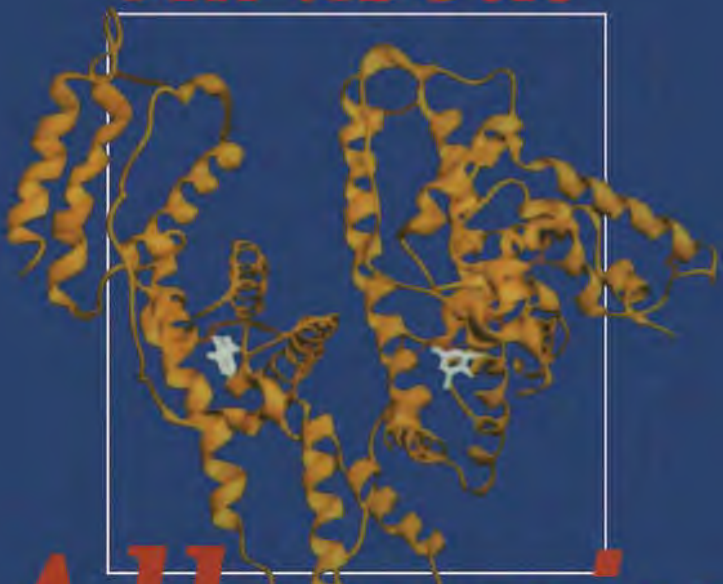
All about Albumin



Biochemistry, Genetics, and Medical Applications

THEODORE PETERS, JR.

All about Albumin

Biochemistry, Genetics, and Medical Applications



A medic administering blood plasma (shortly to be replaced by purified albumin) to a wounded U.S. Soldier in Sicily in 1943. With appreciation to the National Library of Medicine.

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All about Albumin

Biochemistry, Genetics, and Medical Applications

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Front cover photograph: Ribbon diagram of tertiary structure of human albumin as derived by X-ray crystallography. Reproduced from He and Carter (1992) by permission of *Nature*.

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To the thousands of students of albumin who have contributed to our knowledge of this captivating protein over many years. And particularly to my lovely wife and companion of over 50 years, Margaret Campbell Peters, who has supported my efforts and tolerated this rival without jealousy.

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Foreword: All about Albumin

Albumin is one of the longest known and probably the most studied of all proteins. Its manifold diverse functions have attracted the interest of scientists and physicians for generations. Its applications are many, both in clinical medicine and in basic research. Yet, until now, no monograph has been published that brings together the full scope of albumin: its history, structure, physical and biological properties, genetics, metabolism, clinical applications, and its preparations and uses in the laboratory. This book is timely, and no one is more qualified to write it than Dr. Peters. He has spent a lifetime in the study of albumin, and has also been the colleague, advisor, and friend of many whose research has contributed to knowledge of this protein.

Albumin is the most abundant soluble protein in the body of all vertebrates and is the most prominent protein in plasma. Some of its physiological properties have been recognized since the time of Hippocrates; albumin was named and first studied a century and half ago and was crystallized a century ago. Yet, the recent elucidation of its three-dimensional structure depended on crystallization in the space shuttle and recombinant technology. The physiological functions of albumin were the prime incentive for the intensive wartime program of plasma fractionation beginning in 1942 at Harvard. Not only did this program, and its commercial and university affiliates, produce tons of highly purified, stable, virus-free albumin for battlefield use, it also provided the methodology for the purification of many other plasma proteins. In peacetime this led to a national program for blood procurement and plasma fractionation and the development of other products for clinical use such as gamma globulin and clotting factors. The availability of so much pure albumin, at a time when other proteins had to be purified laboriously, made albumin the favorite model for study by protein chemists. This prompted a voluminous increase in literature that has not abated to this day. Only a person with a lifetime of devotion to albumin could put this vast literature into perspective and summarize and interpret it as Dr. Peters has done.

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The structure, properties, and ligand binding of albumin, described in Chapters 2 and 3, are intimately connected and constitute the prime interest of protein chemists, biochemists, and pharmacologists. The repeating pattern of three largely helical domains stabilized by multiple disulfide bonds is unique to the albumin family of proteins. Albumin itself is unique in its myriad affinities. How a protein with only two major binding sites can exhibit such diverse affinities is a puzzle to the protein chemist and a predicament for the pharmacologist. Crystallographic study of the binding of ligands has elucidated the complex nature of the sites in some instances, but many cases remain to be explained.

Unlike many other plasma proteins that exhibit polymorphisms and mutations, some of which are harmful, genetic variants of human albumin are rare and benign; hence, until recently there was little study of albumin mutations. However, after the protein sequence and, later, the gene and genomic sequences discussed in Chapter 4 were determined, identification of the cause of genetic variations of albumin was undertaken. More than 50 mutations have been identified: point mutants, frameshifts, and splicing errors. None of these is pathological, but analbuminemia is paradoxical. How can the very rare persons who essentially lack albumin exhibit only minor symptoms when it has been shown that such important properties as maintenance of blood volume and binding and transport of metabolites and drugs are prototypical properties of albumin? The albumin superfamily, initially comprising albumin, α-fetoprotein, and vitamin D-binding protein, has recently been augmented by the discovery of afamin, or α-albumin. Chapter 4 shows that the structural relationships within this family and the sequence homology of albumins of species ranging from man to the lamprey are providing insights into the evolution of albumin.

The metabolism and clinical aspects of albumin are interrelated but are described separately in Chapters 5 and 6, respectively. As a nonglycosylated single chain of 585 amino acids tightly folded into three domains by 17 intrachain disulfide bonds, albumin offers a revealing model for study of the processes of expression, secretion, and intramolecular folding to produce the mature protein. Prompted by its multiple functions, many investigators have analyzed the mechanism and rate of biosynthesis and the catabolism of albumin, as well as its distribution in the serous fluids of the body. Changes in serum albumin concentration in disease, typically the marked decline in malnutrition and in renal and liver disease, have long served as diagnostic and prognostic criteria. Because of potential viral contamination of blood and plasma, notably hepatitis and AIDS, albumin, which is readily rendered virus-free by heating for 10 hours at 60°C, has been widely used in surgery and in the treatment of shock and trauma. It is this application, approaching 100,000 kg a year in North America alone, that has required large-scale methods of commercial production and has recently prompted the application of recombinant technology. These methods are described in Chapter 7, which also illustrates the many *in vitro* applications familiar to the biochemist and microbiologist.

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My title "All about Albumin"* tells it all. In the many areas described above, this long-needed monograph will serve as a handbook for the experienced investigator and as an invaluable reference source for all who have a need to know about albumin. It deserves a place on the shelves of all libraries of medicine and basic medical sciences, and of biology and chemistry. I recommend it highly to physicians, clinical investigators, biochemists, protein chemists, pharmacologists, biologists, and chemists.

Frank W. Putnam
Indiana University
Bloomington, Indiana

* Author's Note: The publisher and I found Dr. Putnam's title an appealing one and so adopted it, somewhat presumptuously, as the title for the volume. Our thanks to Dr. Putnam.

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Preface

Every student of biology and medicine is familiar with serum albumin as the most abundant and most easily measured protein of blood plasma. One of the purest proteins available commercially at a reasonable cost, albumin is also known to clinicians, nutritionists, physical chemists, biochemists, immunologists, and, more recently, to geneticists and molecular biologists. Medically, a generous concentration of albumin in the bloodstream is a measure of the "Quality of Life" (Kobayashi *et al.*, 1991). The value of its commercial production exceeds one billion dollars annually. Yet there have been few reviews of the chemistry of albumin, and no monographs on this protein other than reports from two conferences. Hence, on retiring from laboratory activity after a lifetime of interest in this intriguing protein, I undertook the somewhat ambitious task of summarizing in one volume its chemistry, genetics, metabolism, clinical implications, and commercial aspects.

This book is intended as a resource for students and practitioners of protein chemistry who use albumin as a model protein for physical or chemical studies, as well as for clinical researchers interested in plasma protein metabolism and in transport of substances in the blood. I hope it will also prove useful to those studying genetic variation, of which much has been learned concerning albumin in recent years, and to molecular biologists who use albumin as a paradigm for elucidating the mechanisms of genetic activation and control. The largest group to whom this book may prove of value, however, are those who do not study albumin but use it for its beneficial properties. I refer to the surgeons who administer albumin intravenously to bolster the failing circulation of their patients, or the nutritionists who give albumin to promote intestinal function so that their patients may eat again. I refer also to the many workers in academic, medical, and industrial laboratories who include albumin as an essential component of the supporting medium of their cell cultures or who add albumin *in vitro* to protect delicate macromolecules from adsorption to the surfaces of containers. I hope that each of these groups will find some information pertinent to their

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particular application and will delve a bit into the other sections of this book so they may gain a better overall appreciation of the properties and mysteries of the protein they are using. They may then be better prepared to understand the functioning of albumin in the system under study and perhaps to understand the functioning of the system itself.

Various problems arise in research systems from inadequate knowledge of the properties of albumin. When it is added as a support protein to avoid effects of the surface of a container on enzymes or cells, its avidity for fatty acids and metals may affect the system's performance. Commercial albumin preparations have all been heated to 60° (the degree symbol refers to degrees Celsius throughout this book), which can cause subtle changes in its tertiary structure; users should also be aware that octanoate or *N*-acetyltryptophan are commonly added to protect albumin from denaturation on heating, and traces of these ligands remain unless removed by special treatment. Smidgens of granulocyte proteases, of insulin, and of α_1-antitrypsin may copurify with albumin and cause strange results in cell cultures. I hope that this book, without being overwhelmingly technical, can at least assist in a more-informed application of this protein.

My own familiarity with albumin arose, by chance, quite early in my adventures in biochemistry while a graduate student in the laboratory of Christian B. Anfinsen at Harvard Medical School. This was in the immediate post-World War II period—radioisotopes of convenient half-life had just become available as a by product of the atomic pile. Only four years earlier, my college biochemistry course had categorized proteins among the colloids. While measuring the incorporation of ^{14}C into the proteins of a chicken liver slice system, it became apparent after many hours of fractionation in a subzero cold room that the most highly labeled protein in the system was one which had been secreted into the incubation medium. Naively, perhaps, I already believed that the homogeneous, soluble protein of about 70,000 Da was albumin, but the skeptical Dr. Anfinsen was only convinced by a beautiful white immune precipitate which formed before our eyes when an antiserum to chicken serum proteins was added.

The proximity of the Harvard Physical Chemistry Laboratory under Edwin J. Cohn provided advisors such as John T. Edsall and Douglas M. Surgenor on the properties of albumin. It also provided the imposing E. J. Cohn himself as the chairman for my thesis defense, a daunting experience for an awestruck graduate student. This group (see Chapters 1 and 7, and particularly Fig. 1-1) had just completed the major wartime effort of the American Red Cross blood fractionation program to provide human albumin as a stable substitute for blood plasma for wounded soldiers on the battlefield. A later visit to the Carlsberg Laboratorium directed by the eminent protein chemist Kai Linderstrøm-Lang helped me gain an appreciation of the sturdiness and resiliency of the albumin molecule. This appreciation grew during a period of more than three decades in the laboratories of The Mary Imogene Bassett Hospital, a forward-looking insti-

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tution in rural upstate New York in which I was encouraged in my pursuit of this protein without interference. Here I was joined by colleagues Richard C. Feldhoff and Roberta G. Reed, who were likewise intrigued by albumin and who have continued its study, Dr. Reed at the Bassett Hospital and Dr. Feldhoff at the University of Louisville.

One of the joys of this pursuit has been meeting and exchanging ideas with leading students of albumin biochemistry. In many cases they have openly furnished unpublished information and made suggestions without personal gain. While the names of colleagues appear throughout this volume, I would like to thank in particular for their friendship and, frequently, hospitality Leon L. Miller, Peter N. Campbell, Julian B. Marsh, J. D. Judah, Gerhard Schreiber, Hans Glaumann, Colvin Redman, and Marcus A. Rothschild, researchers in albumin biosynthesis; Margaret J. Hunter, Walter L. Hughes, Joseph F. Foster, Frank R. N. Gurd, Claude Lapresle, T. P. King, R. H. McMenamy, James R. Brown, B. Meloun, Arthur A. Spector, Rolf Brodersen, and Ingvar Sjögren, who studied its chemistry; and Franco Porta, Andrew Tárnoky, Stephen O. Brennan, Monica Galliano, Frank W. Putnam, Achilles Dugaiczyk, D. R. Schoenberg, and Luc Bélanger, pioneers in the study of its genetic makeup.

I hope that I have treated the reports of all of these albumin colleagues fairly in this book; it was certainly my intent. In addition, I express my thanks to several who helped in its preparation: John S. Finlayson of the U.S. Food and Drug Administration, who has kept an eye on commercial albumin preparations for years, and Jean A. Thomas and Timothy Tiemann of Miles Laboratories, Inc., who have helped me understand the complexities of the commercial production of a protein in bulk. The willing help of the medical librarians of the Bassett Hospital, Linda Muehl and Robin Phillips, has been invaluable. I would be remiss to conclude without a word of appreciation to the many kind people who have stimulated and encouraged me in the world of science: Christian B. Anfinsen, Eric G. Ball, and A. Baird Hastings of Harvard Medical School; Joseph W. Ferrebee, James Bordley, III, Clinton V. Z. Hawn, Charles A. Ashley, Roberta G. Reed, Gary A. Weaver, and Eugene W. Holowachuck of the Bassett Hospital; and also John H. Powers of that institution, who always urged me to write this book. For encouraging my curiosity at an earlier age, I owe a large debt to my mother, Miriam Lenhardt Peters, and to my first instructor of rigorous science, Susie Kriechbaum, high school geometry teacher. My own advice to students seeking a career in biological research has consistently been: Study mathematics and logic and the great science of chemistry. Then you will be better able to understand the marvelous mechanisms of life. And I hope the pursuit will be as enjoyable and exciting for you as it has been for me.

Theodore Peters, Jr.

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List of Abbreviations

aa	Amino acid; one-letter code given with Fig. 2-9
AFP	α-Fetoprotein
AFM	Afamin (Lichenstein *et al.*, 1994)
A/G	Albumin/globulin ratio in serum
ALF	α-Albumin (Bélanger *et al.*, 1994)
ANS	1-Anilino-8-naphtho-sulfonic acid
BCG	Bromcresol green
BCP	Bromcresol purple
BMA	Bovine mercaptalbumin
bp	Nucleotide base pair
BSA	Bovine serum albuimin
BSP	Sulfobromophthalein
BW	Body weight
CD	Circular dichroism
cDNA	Copy DNA (from mRNA)
CMPF	3-Carboxyl-4-methyl-5-propyl-2-furanpropanoic acid
COP	Colloid osmotic pressure, also oncotic pressure
Da	Daltons
DBP	Vitamin D-binding protein, also Gc-globulin
DE	Distal element (genetics)
DEAE	Diethylaminoethyl
DNP	Dinitrophenyl
DTNB	Ellman's reagent, 5,5'-dithiobis(2-nitrobenzoic acid)
EGF	Epithelial growth factor
ER	Endoplasmic reticulum
ESR	Electron spin resonance
EV	Extravascular
FDH	Familial dysalbuminemic hyperthyroidism
FDNB	Fluorodinitrobenzene
FTIR	Fourier-transform infrared
GC	Gas chromatography
GFR	Glomerular filtration rate
GI	Gastrointestinal
GRE	Glucocorticoid receptor element (genetics)
GuCl	Guanidinium chloride
h	Hour
HABA	2-(4'-Hydroxyphenylazo) benzoic acid
HBV	Hepatitis B virus

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HIV	Human immunodeficiency virus
HMA	Human mercaptalbumin
HPLC	High-performance liquid chromatography
HSA	Human serum albumin
IDDM	Insulin-dependent (Type-I) diabetes mellitus
Ig	Immunoglobulin
IL	Interleukin
IR	Infrared
kb	Kilobase
kDa	Kilodaltons
L	Liter
LCFA	Long-chain fatty acids, C_{16}–C_{20}
M	Moles/liter
MCFA	Medium-chain fatty acids, C_6–C_{14}
M/M	Mole/mole
MMADS	Monoacetyldiaminophenyl sulfone (bilrubin analog)
mRNA	Messenger ribonucleic acid
My	Million years (evolution)
NAR	Nagase analbuminemic rat
NASA	National Aeronautic and Space Agency
NIDDM	Non-insulin-dependent (Type-II) diabetes mellitus
NMR	Nuclear magnetic resonance
NSA	Normal serum albumin (commercial fraction V for IV use)
ODMR	Optically detected magnetic resonance
ORD	Optical rotatory dispersion
PCR	Polymerase chain reaction
PE	Proximal element (genetics)
PEG	Polyethylene glycol
RER	Rough-surfaced endoplasmic reticulum
RFLP	Restriction-fragment length polymorphism
RSA	Rat serum albumin
s	Second
SE	Sulfoethyl
T3	Triiodothyronine
T4	Thyroxine
TCA	Trichloroacetic acid
TNF	Tumor necrosis factor
tRNA	Transfer RNA
UV	Ultraviolet
°	Degree Celsius

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1

Historical Perspective

The name albumin evolved from the more general term, *albumen*, the early German word for protein. Its origin was Latin, *albus* (white), the color of that part of an egg surrounding the yolk when it is cooked. Album*en* is still used for the white of an egg, for the secretion of the snail, and for urinary proteins as a group, whereas the -in ending refers to the specific protein from blood plasma or to a protein with similar properties.

Albumin, hemoglobin, and fibrin were probably the first proteins of the body to be studied. The Greek physician Hippocrates of Cos noted in his *Aphorisms* that a foamy urine, in all likelihood caused by the presence of albumin, indicates chronic kidney disease. The Swiss physician Paracelsus in the sixteenth century caused protein to precipitate from urine with vinegar; near the end of the eighteenth century Frederick Dekkers obtained the same result by heating.

When Harvey described the circulation of the blood in his lectures in 1616, chemists of the day were acquainted with blood serum as the fluid that extrudes from clotted blood as it contracts on standing. They recognized that serum contained protein, or "albumen." H. Ancell noted in his lectures in England in 1837, as cited by several reviewers, that "albumen is doubtless one of the most important of the animal proximate principles: it is found not only in the serum of the blood but in lymph, chyle, in the exhalation from surfaces, in the fluid of cellular tissue, in the aqueous and vitreous (humors) of the eye, in many other animal fluids." Because no fractionations of the proteins had been reported, by "albumen" Ancell was actually referring to the total protein of these fluids.

The French physiologist, C. Denis, in 1840 performed the first recorded dialysis by placing blood serum in a sac of intestine immersed on water; he found that some of the protein precipitated as the salt was removed through the sac.

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Unlike the action of heat, this precipitation was reversible when small amounts of salt were added. The protein soluble in water without salt was called albumin and that which precipitated in little globules, globulin. The term albumin still is used operationally to refer to a protein that is soluble in distilled water saturated with carbon dioxide; it includes plant albumins and the ovalbumin of egg white.

Early protein chemists also used salt as a precipitating agent. Saturation of blood serum with ammonium sulfate, the most effective salt then available, caused the reversible precipitation of protein. In the late 1800s, the Swiss pharmacologist, G. Kander, showed that the protein that precipitated from blood serum on half-saturation with ammonium sulfate corresponded to the globulin precipitated by dialysis, and the soluble protein that remained corresponded to albumin. Actually, more globulin is precipitated by this salt treatment than on dialysis; the globulin precipitating with dialysis was termed euglobulin, or true globulin, and that remaining soluble on dialysis but precipitating with salt was termed pseudoglobulin. The albumin obtained by half-saturation with ammonium sulfate is thus more pure than that obtained by dialysis.

Salt fractionation was the first method used by clinical chemists to study the protein composition of blood serum in a medical setting. Because the protein was determined by the Kjeldahl analysis for total nitrogen, sodium sulfate was substituted for ammonium sulfate. P.E. Howe in 1921 published a procedure that was the standard method for three decades for serum protein assay, using sodium sulfate kept at 37° for greater solubility of the salt. The ratio of soluble to precipitated protein became the albumin/globulin ratio, or A/G ratio, which is still useful as a rough index of health (see Chapter 6, Section II,A).

Chemists of the nineteenth century had refined crystallization to an art, and albumin was one of the earliest proteins they attempted to crystallize. A. Gürber in 1894 obtained horse albumin as crystals by bringing an aqueous solution to its isoelectric point, pH 4.9. Paradoxically, although crystallization is used as a criterion of purity, the albumin molecule is so flexible and includes so many adherent compounds that crystallization does not yield as pure a product as do other fractionation procedures (Chapter 7, Section I).

Crystallization did at least give investigators confidence that albumin, unlike the globulin fraction, is a single, reproducible substance. The only other method then available to determine purity of a protein preparation, demonstration of a sharp break in the solubility curve as the protein concentration was increased (Herriot, 1942), has never been satisfactorily applied to serum albumin.

T. Svedberg and K. O. Petersen in Uppsala studied the proteins of blood serum in the 1930s by the new technique of ultracentrifugation. They found three primary bands of sedimentation velocity 4S, 7S, and 19S. The 4S band, having an approximate molecular mass of 70,000 Da, was albumin. By the homogeneity of the bell-shaped Schlieren peak it was possible to judge the purity of an albumin preparation.

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When A. Tiselius, in the Svedberg laboratory in 1937, applied the Schlieren optics developed by Svedberg to the technique of electrophoresis, albumin was readily identified as the prominent anionic constituent, whereas the globulin fraction was separated into three bands, which he termed α, β, and γ. Putnam (1993) has described these exciting times in the life of Tiselius. Later, use of barbiturate in place of phosphate buffers revealed two bands in the α-1 and α-2, and use of solid supports such as agarose gel caused the β band to resolve into several components as well.

Electrophoresis has continued to be the major method for identifying and judging the purity of albumin preparations. Except for trace constituents of like electrophoretic mobility, such as insulin and amylase, the albumin band on electrophoresis of blood serum is essentially a single species of protein, which we term serum albumin, or just albumin. Plasma albumin is the same protein; the term arose from the use of blood plasma rather than serum as a more productive source for commercial fractionation (see Chapter 7, Section I.B) and is more frequently heard in commercial circles or among protein chemists trained in the early days of plasma fractionation.

Table 1-1 lists the chronology of events related to our current knowledge of the albumin molecule. It touches on structure, genetics, metabolism, clinical applications, and commercial production, topics that are expanded in the succeeding chapters.

The greatest impetus to the preparation of albumin as a pure protein came during World War II, when a critical need for a stable substitute for blood plasma on the battlefield resulted in the development of the cold alcohol fractionation procedures by E.J. Cohn and colleagues at the Plasma Fractionation Laboratory of the Harvard Department of Physical Chemistry (Fig.1-1). Albumin was selected in 1940 by the Subcommittee on Blood Substitutes of the Committee on Transfusion as being more stable, less antigenic, and less viscous than whole plasma (Coates and McFetridge, 1964). The Harvard laboratory in 1940 established procedures to purify albumin from bovine plasma by the cold ethanol method still widely in use. The major advances were the ability to remove the solvent by evaporation at low temperatures, avoidance of addition of salts, and suppression of growth of bacteria during processing.

Unfortunately, bovine albumin was first employed owing to the availability of bovine plasma in large quantity. As might in retrospect have been predicted, but was not realized in the early 1940s, bovine albumin given intravenously caused serum sickness in some of the volunteer subjects, resulting in at least two deaths from kidney failure. Even crystallization to purify the albumin and remove all but 0.008% of globulins was ineffective. On 22 March, 1943, the official bovine albumin program ended.

Recognizing that it was species differences, not impurities, that caused the severe reaction to bovine albumin, the emphasis of the plasma substitute program

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TABLE 1-1
Chronological History of Serum Albumin

Year	Source	Comments	Reference
400	Hippocrates	Noted foam on urine with renal disease	Hippocrates (1978)
1500	Paracelsus	Precipitated protein from urine with vinegar	Pagel (1982)
1616	Harvey	Described circulation of blood	Harvey (1628)
1790	Dekkers	Precipitated protein from urine with heat	Major (1945)
1837	Ancell	Lectured on distribution of protein in body	Ancell (1839)
1840	Denis	Separated "albumin" with dialysis	Denis (1859)
1886	Kander	Separated albumin with ammonium sulfate	Kander (1886)
1894	Gürber	Crystallized horse albumin	Gürber (1895)
1896	Starling	Presented role of albumin in maintaining circulation	Starling (1909)
1921	Howe	Devised clinical albumin/globulin assay with sodium sulfate	Howe (1921)
1923	Bennhold	Showed binding of Congo Red by albumin *in vivo*	Bennhold (1923)
1924	Kekwick	Established purity of an albumin preparation	Kekwick (1938)
1926	Svedberg	Measured molecular mass with ultracentrifuge	Svedberg (1934)
1932	Race	Separated albumin with acid acetone	Race (1932)
1934	Hewitt	Crystallized human albumin plus long-chain fatty acid	Hewitt (1936)
1937	Tiselius	Separated albumin by electrophoresis	Tiselius (1937)
1938	Kabat	Found albumin molecule to be elongated	Kabat (1938)
1939	Luetscher	Detected N → F isomerization in weak acid	Luetscher (1947)
1940	Cohn	Prepared bovine and then human albumin for intravenous use	Cohn (1941)
1946	Cohn	Published commercial fractionation scheme with cold ethanol	Cohn *et al.* (1946)
1947	Hughes	Crystallized human albumin mercury dimer	Hughes (1954)
1947	Klotz	Studied effect of albumin on structure of bound dyes	Klotz *et al.* (1948)
1950	Peters	Noted biosynthesis of albumin in chick liver slices	Peters and Anfinsen (1950a)
1951	Sterling	Used ^{131}I-labeled albumin to measure turnover	Sterling (1951)
1954	Miller	Demonstrated biosynthesis of albumin in perfused rat liver	Miller *et al.* (1951)
1954	Bennhold	Reported first two cases of analbuminemia	Bennhold *et al.* (1954)
1956	Sober	Separated albumin by ion-exchanged chromatography	Sober *et al.* (1956)
1957	Knedel, Nennstiel	Reported first cases of genetic bisalbuminemia	Knedel, Nennstiel and Becht (1957)
1960	Foster	Studied isomeric forms; proposed "domain" type of structure	Foster (1960)
1961	Campbell	Showed albumin formation by rough endoplasmic reticulum	Campbell and Kernot (1962)
1969	Bowman	Noted similarity of vitamin D-binding protein to albumin	Bowman (1962)

(*continues*)

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TABLE 1-1—*Continued*

Year	Source	Comments	Reference
1970	King	Studied tryptic fragment of bovine albumin	King and Spencer (1970)
1971	McMenamy	Studied cyanogen bromide fragments of human albumin	McMenamy *et al.* (1971)
1973	Judah, Schreiber	Detected proalbumin in rat liver	Judah *et al.* (1973) Urban *et al.* (1974)
1975	Brown	Deduced amino acid sequence of bovine albumin	Brown (1975)
	Meloun	Deduced amino acid sequence of human albumin	Meloun *et al.* (1975b)
1976	Ruoslahti	Showed homology of α-fetoprotein to albumin	Ruoslahti and Engvall (1976)
1977	Strauss	Reported signal peptide sequence of rat prepralbumin	Strauss *et al.* (1977)
1979	Sargent	Isolated gene for human albumin	Sargent *et al.* (1979)
1981	Lawn	Reported base sequence of human albumin cDNA	Lawn *et al.* (1981)
1986	Dugaiczyk	Reported complete gene sequence of human albumin	Minghetti *et al.* (1986)
1989	Brennan, Putnam, Galliano	Studied locations of mutations in albumin molecule	See Table 4-8, in Chapter 4
1992	Carter	Found heartlike crystal structure of human albumin	He and Carter (1992)
1994	Bélanger, Lichenstein	Reported cDNA sequence of α-albumin and afamin	Bélanger *et al.* (1994), Lichenstein *et al.* (1994)

quickly switched to production of human albumin. Purification of albumin from human plasma had begun in 1941, using blood provided by the American Red Cross. Surgeon I.S. Ravdin, now in the uniform of a general, administered nearly the entire available stock to seven severely burned sailors after the 7 December, 1941, attack on Pearl Harbor; all seven survived. The program was then greatly expanded. Using the technique developed in the laboratory of Cohn, first Armour, then Lederle, then a total of seven commercial laboratories produced nearly 600,000 12.5-g units of albumin from over 2 million units of blood. The human albumin contained less than 2% globulins, and was packaged as a 25% solution to save space; at this concentration it was noted to be "isoviscous" with whole blood. Merthiolate (thimerosal) was included as a preservative. In 185 injections into volunteers there were no reactions of any kind; to this day there have been no cases of transmission of viral disease from properly prepared commercial albumin.

"Albumin" thus became a cry by medical personnel on the battlefield (see Frontispiece). By 1945 the specifications had been modified to allow 3% globulins,

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Fig. 1-1. Members of the Department of Physical Chemistry of the Harvard Medical School, 1944, at the peak of the effort to produce pure human albumin for battlefield use. Front row, left to right: **J. Edsall, Bailey, Oncley, Janeway, E.J. Cohn, Scatchard,** Enders, **Strong,** Ingraham; second row: **Woodruff, Brown, Sparrow, Hughes,** Williams, G. Edsall, Ferry, Reichert, **Mulford,** Deutsch, Bering; third row: Klein, Goodrum, Larson, **Morrison,** Gordon, Gross, **Foster,** Aldrich, Berenberg, Taylor; fourth row: French, O'Neil, Blanchard, **Ashworth,** Dale, Miller, Brooks, Barker, Gahm, Griffith; fifth row: Sanderson, Fleming, Cameron, Poole, Traill, Weeks, Smith, Beauchamp, Sullivan, **Hasson;** top row: Whitaker, Splaine, O'Reilly, Kottke, **Budka.** (Bold face indicates authors cited in this volume.) Reproduced from Cohn (1946) with appreciation to the National Library of Medicine.

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with pasteurization in its final container substituted for mercurial preservative. As an offshoot of its ready availability in bulk after the war, its intravenous use was continued by surgeons and nutritionists and albumin became a major item in hospital pharmacies (see Chapter 6, Section III).

A further benefit of the commercial availability of this highly pure protein was the adoption of albumin as a model by protein chemists. Volumes of data on its molecular characteristics were reported by former members of the E.J Cohn group (Fig. 1-1), among them G. Scatchard, J.T. Edsall, J.L. Oncley, W.L. Hughes, L.A. Armstrong, F.R.N. Gurd, J.F. Foster, and M.J. Hunter, as well as by other physical chemists such as C. Tanford, W. Kauzmann, J. Steinhardt, G. Weber, M. Laskowski, L.O. Andersson, D.W. Wetlaufer, K. Aoki, A. Shrake, and D.C. Benjamin. The interplay with ligands was studied by H. Bennhold, I.M. Klotz, A.A. Spector, H. Ott, D.S. Goodman, B. Sarkar, R.H. McMenamy, C. Jacobsen, R. Brodersen, G. Blauer, I. Sjögren, U. Kragh-Hansen, and R.G. Reed. (Many of these workers are personal acquaintances who have been mentioned in the Preface. References to their publications and those in the following paragraph will be found in the bibliography at the end of this volume, as well as in specific chapters.)

At the same time other groups were investigating the metabolism of albumin: P.N. Campbell, L.L. Miller, G. Schreiber, J.D. Judah, C.M. Redman, and M.A. Rothschild. The amino acid sequence of albumin appeared from the laboratories of Brown and Meloun in 1975, somewhat late among well-known proteins owing to the length of the single peptide chain but very welcome nonetheless. In the past 10 years the nature of the albumin messenger RNA and gene has been determined through the efforts of workers such as R.M. Lawn, S.M. Tilghman, A. Dugaiczyk, and R.F. Doolittle. A tertiary structure has been produced by D.C. Carter and J.X. Ho, building on earlier X-ray diffraction efforts by B.W. Low, B.M. Craven, A. McPherson, and others, and yielding some surprises to those predicting a smooth, cigarlike molecule on the basis of hydrodynamic studies. A fourth member, termed α-albumin, or afamin, has joined the albumin superfamily of albumin, α-fetoprotein, and vitamin D-binding protein (Chapter 4).

A selected group of review articles that cover the biochemistry of albumin over the past 70 years would include Cohn (1925, 1941), Hughes (1954), Foster (1960), Watson (1965), Schultze and Heremans (1966), Peters (1975, 1985), Rosenoer *et al.* (1977), Roche (1986), and Rothschild *et al.* (1988). Reviews dedicated to specific aspects, such as ligand binding, are listed in the pertinent chapters.

There is much that we understand about albumin biochemistry: the basis for its flexibility and stability (presumably inherent in its triple-domain structure with numerous disulfide bonds), the location of some of its binding sites, the steps of its biosynthesis, and the cause of the rare and intriguing condition of analbuminemia in rats and humans. Yet there is much left to learn. What are the details of its tertiary structure, in solution as well as in crystals? What is the

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function of the amino-terminal hexapeptide found in the precursor molecule, proalbumin? What are the mechanisms of control of albumin biosynthesis? Where are the evolutionary origins of this protein, which can only be traced with surety to the lower chordates?

Is this protein essential? How can a handful of humans and rodents almost totally lacking albumin survive to late adulthood and even reproduce? After the formal report of the first two recognized cases of analbuminemia at Tübingen in 1954, a story is told that colleagues of the albumin investigator Professor H. Bennhold approached to offer him condolences on the demise of the importance of his protein. Subsequent studies have shown that many of the functions performed by albumin can be assumed by other plasma proteins, but there remain disturbances of lipid metabolism in its absence, and there may be unrecognized physiological roles of the trace amounts of albumin that are invariably present. One of the simplest yet most perplexing questions about albumin, its physiological function, thus remains.

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2

The Albumin Molecule: Its Structure and Chemical Properties

This chapter concentrates on structural information about human and bovine albumins: human albumin for its obvious importance in clinical, metabolic, and genetic studies, and bovine albumin for its role as a model protein and its many *in vitro* applications. Amino acid composition and sequence data available on other species, e.g., the rat, mouse, pig, horse, sheep, chick, snake, frog, fish, and lamprey, are given in Chapter 4.

I. PRIMARY STRUCTURE AND COMPOSITION

A. Amino Acid Sequence and Disulfide Bonding Patterns

Sequences and disulfide bonding patterns of human and bovine albumins are shown in the three-letter amino acid code in Figs. 2-1 and 2-2, respectively. The sequences are derived from complementary DNA (cDNA) data, whereas the disulfide bond locations and the orientation of loops are derived from peptide studies by Saber *et al.* (1977) and Brown (1974). The loop orientation does not accurately represent the arrangement in a crystal as seen by X-ray diffraction (discussed later in this chapter), but is consistent with hydrodynamic properties of albumin and is useful in displaying the molecule in a linear fashion to visualize the homology among domains.

There are nine (actually eight and one-half) double loops formed by disulfide bonds involving adjacent half-cystine residues. These can be further grouped

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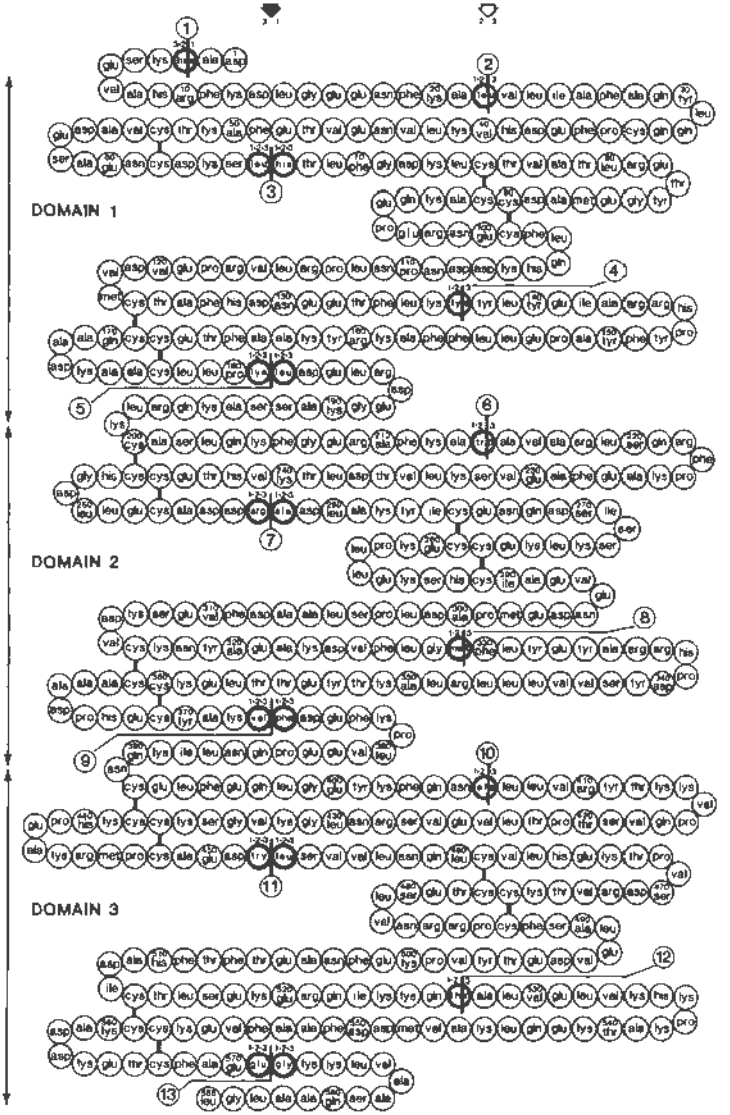


Fig. 2-1. Amino acid sequence of human serum albumin, derived from the cDNA sequence of Minghetti *et al.* (1986). The general layout of the molecule corresponds to the linear model (Fig. 2-5) compatible with the hydrodynamics of albumin in solution rather than that derived from X-ray diffraction studies (Fig. 2-7), because it allows the internal homology to be appreciated more readily. The numbered vertical bars indicate the positions of introns in the pre-mRNA. Reproduced by permission of *The Journal of Biological Chemistry*.

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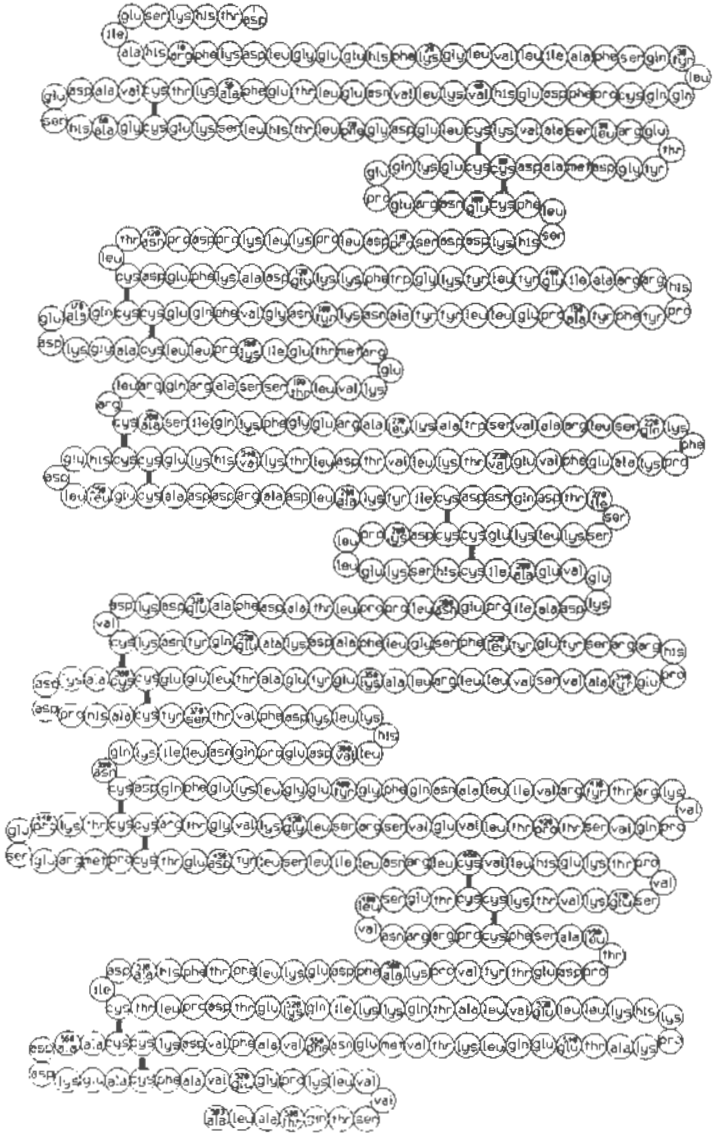


Fig. 2-2. Amino acid sequence of bovine serum albumin, derived from the cDNA sequence of Holowachuk (Holowachuk, 1991). Thr was substituted for Ala-190 to agree with the reported chemical sequences (Brown, 1975; Hirayama *et al.*, 1990). Note that there is a gap at the position corresponding to residue 116 of HSA, so that the numbers of all residues beyond that position should be increased by 1 when comparing to sequences of other species.

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into three homologous domains, each containing two longer loops separated by a shorter loop. All known serum albumins of vertebrates evolutionarily higher than reptiles have the same distribution of half-cysteines and presumably this same loop–domain structure; the related proteins, α-fetoprotein, α-albumin, and vitamin D-binding protein (Chapter 4, Section II), contain most of it.

The homologous domains are usually numbered I, II, and III, counting from the amino terminus. A similar architecture of assembly from similar domains is common with plasma proteins, for example, transthyretin (thyroxine-binding prealbumin) and transferrin, and has been proposed as a convenient evolutionary mechanism of providing a protein that is large enough to avoid spillage from the circulation through the kidney. In the case of albumin, at least, the three domains, although homologous, have different ligand-binding functions. Within each domain the first two loops, loops 1–2, 4–5, and 7–8, are grouped together as subdomains IA, IIA, and IIIA, respectively, and loops 3, 6, and 9 are called subdomains IB, IIB, and IIIB. This grouping has functional significance that is apparent in the X-ray-derived structure (Section II,A).

1. Background of Sequence Determination

Although amino acid sequences of proteins are now readily (and more accurately) derived from cDNA base sequences, the long trail of efforts in sequencing albumin by chemical means deserves to be recorded. A single amino-terminal residue, Asp, was first reported for bovine, human, horse, and pig albumins with Sanger's fluorodinitrobenzene (FDNB) procedure (Desnuelle and Rovery, 1951). Some studies found small amounts of dinitrophenylcystine as well, plus additional amino-terminal residues after oxidation of the disulfide links with perchloric acid. That these albumins were indeed single-chain proteins was established only when Thompson (1958) showed that careful oxidation of the 17 cystine links of bovine albumin did not cause any more amino-terminal groups to appear. His finding was confirmed when Hunter and McDuffie demonstrated in the following year (1959) that there was no change in molecular mass of human albumin determined by light scattering or ultracentrifugation after careful reduction of all of its disulfide bonds in sodium dodecyl sulfate (SDS) and alkylation of the resultant thiols with iodoacetamide. The additional amino-terminal residues found on oxidation in the earlier studies were probably the result of peptide bond cleavage by the acidic conditions employed, and of small amounts of cystine shown by King (1961) to be bound as a mixed disulfide (Section I,B).

2. Human Serum Albumin

The peptide sequence of human albumin was known before its cDNA sequence. The complete sequence was reported almost simultaneously by Brown and co-workers in Austin, Texas (Behrens *et al.*, 1975) and Meloun *et al.* in

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Prague (1975b). Other laboratories had laid groundwork. In 1954 Thompson reported the first results with the Edman degradation: Asp-Ala for human and Asp-Thr for bovine albumins (1954). In 1955 White *et al.* found the four-residue C-terminal sequence with carboxypeptidase. Witter and Tuppy (1960) first described the sequence 31–36, containing the single cysteine; Sugae and Jirgensons (1964) described residues 214–217, containing the single tryptophan; Bradshaw and Peters sequenced residues 1–24 (1969); Swaney and Klotz (1970) expanded the sequence around the tryptophan to 212–218.

The Meloun group worked with fragments obtained by cleavage at methionyl residues with cyanogen bromide. Of the six methionine residues in human albumin, two are in the peptide links outside of disulfide loops, at residues 123 and 298. Hence three fragments can be produced without cleaving disulfide bonds. McMenamy *et al.* (1971) named these A (299–585), B (1–123), and C (124–298) for the order in which they were eluted from his column; their position in the albumin chain was not as yet known. After reduction of the S–S bonds additional fragments result from cleavages at methionyl residues 87, 329, 446, and 548. The Meloun group used all seven of these cyanogen bromide-derived fragments. Their results were reported as follows: residues 549–585 (Meloun and Kušnír, 1972); 299–329 (Kušnír *et al.*, 1973); 88–123 (Kušnír and Meloun, 1973); 1–87 (Meloun *et al.* 1975a); 330–446 (Kostka *et al.*, 1975); 447–548 (Morávek *et al.*, 1975; Meloun and Morávek, 1977); and 124–298 (Morávek and Meloun, 1975). Other cyanogen fragment sequences reported were 124–235 (Gambhir *et al.*, 1975), 299–329 (Babin and Goos, 1973), and loop 9 (Walker, 1976b). Bellon and Lapresle (1975) determined the order of some of the cyanogen bromide fragments, a task completed by Meloun *et al.* (1975b).

Two laboratories have reported cDNA base sequences for human albumin: Lawn *et al.* in 1981 and Dugaiczyk *et al.* in 1982. These differed by Lys for Glu-396 and Gly for Glu-97, respectively, from the Meloun and Brown results, and from the later Dugaiczyk result in Fig. 2-1. The cDNA sequences were determined on genetic material from single subjects and could represent either chance allotypy or artifacts in the nucleotide base sequence. The only corrections to the Meloun chemically derived sequence of human albumin from the cDNA-derived sequences were to change Glu-Gln 94-95 to Gln-Glu, Glu-170 to Gln, His-364 to Ala, and Gln-501 to Glu. Considering that the albumin chain of 585 residues was the longest peptide chain sequenced up to that time (1975), this accuracy is a tribute to the care and thoroughness of the work of the Meloun group.

3. Bovine Serum Albumin

As with human albumin, the complete sequence of bovine serum albumin (BSA) was known before its cDNA structure was reported. The chemical sequence is chiefly the work of Brown and co-workers (Brown, 1974, 1975), and dates back to 1969 (Brown *et al.*, 1969). His group worked largely with tryptic

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peptides. Although the details of their work have never been published, their latest sequence (Brown and Shockley, 1982) has been largely confirmed by cDNA sequencing (Fig. 2-2), mass spectrometry (Hirayama *et al.*, 1990), and homology with other albumin sequences (see Chapter 4, Fig. 4-3).

Bovine albumin contains but four methionine residues, at positions 87, 184, 445, and 547. All but Met-184 occur in similar positions in human albumin, and only this methionine lies outside of the disulfide loops. Following its cleavage, fragments N (1–184) and C (185–583) are easily obtained (King and Spencer, 1970; McMenamy and Wesolowski, 1972; Goossens *et al.*, 1973).

Partial sequences of the bovine albumin chain have been reported by other groups in addition to those mentioned with human albumin in the previous section: the C-terminal Ala using hydrazine or reduction with lithium borohydride (Schmid *et al.*, 1959); peptide 1–24 (Shearer *et al.* 1967); three cyanogen bromide fragments, 1–87, 548–583, and 88 184 (King and Spencer, 1972); the order of residues 469–470 (Weijers, 1977); residues 401–404, including an insertion at 401, and amide assignments at 389, 390, 392, and 393 (Reed *et al.*, 1980); and the insertion of Tyr-155 (Ueno *et al.*, 1985).

Weijers (1977) corrected Brown's sequence at five sites. The two insertions bring the total residues in bovine albumin to 583. Residue numbers of BSA in this volume have been altered to include the two insertions and accordingly may be one or two numbers higher than those in earlier reports.

The bovine, pig, sheep, and horse sequences are unique among the albumin sequences reported to date (see Fig. 2-2 and Chapter 4, Fig. 4-3) in having an internal deletion, occurring at residue 116 of human albumin. Hence residue numbers subsequent to 116 in these ungulate species should be increased by one to correspond to albumins of other genera, except in alignments such as that in Fig. 4-3 (Chapter 4), where the deletion is included. All four are also missing the carboxy-terminal amino acid (residue 585) of human albumin, giving them a total of 583 residues.

4. Rat Serum Albumin

A complete peptide sequence of rat albumin has never appeared; Isemura and Ikenaka (1978) had completed the sequence of cyanogen bromide fragments accounting for about one-half of the chain when the cDNA data were reported (Chapter 4, Fig. 4-3) and have apparently not pursued this work further. Agreement between the two sequences is excellent.

B. Composition

The amino acid compositions of human, bovine, and rat albumins derived from their cDNA sequences are tabulated in Table 2-1. (Amino acid composi-

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tions of 16 other albumins are listed in Chapter 4 in Table 4-5.) The most unique feature of the amino acid composition of albumins is the low content of tryptophan, only one or two residues per molecule in mammalian albumins. Compared to a "typical" protein, listed in Table 2-1 as the average of all protein sequences known in 1987, methionine is also characteristically low in these mammalian albumins, as are glycine and isoleucine. Cystine, leucine, and the ionic amino acids, glutamic acid and lysine, are abundant. The large number of ionized residues gives albumins a high total charge, typically 185 ions per molecule at pH 7, which aids its solubility. The acidic amino acids outnumber the basic ones, resulting in a negative net charge at pH 7 of −12 to −17 for the three albumins listed.

Albumin, at least as it is synthesized in the liver, is a simple protein, made up only of amino acids, without prosthetic groups or other additives. Thus it is not a glycoprotein, being one of the few plasma proteins devoid of carbohydrate groups; transthyretin is the other major one. Even albumin ancestors and cousins in the albumin superfamily (Chapter 4, Section II) are glycosylated. Modern albumins generally lack the Asn-X-Ser/Thr sequence needed for N-glycosylation. In three human albumin variants (Chapter 4, Table 4-8), mutation has created the site Asn-X-Ser/Thr and the variant allele is normally glycosylated, without apparent effect on its function. Rat albumin has the triad Asn-Pro-Thr (residues 130–132), but the proline prevents this site from being a functional signal for glycosylation. The amphibian *Xenopus* has two forms of albumin; one is glycosylated and the other is not, whereas the large lamprey albumin is normally glycosylated (Chapter 4, Section III).

In the circulation albumin does accumulate some glucose through nonenzymatic glycosylation as well as small amounts of other tightly bound substances (discussed more fully with the thiol group in Section II.B.5, with ligands in Chapter 3, and with diabetes in Chapter 6, Section II.B.3). Some of the mixed disulfide stowaways are amino acids (cysteine and glutathione); unless they are removed by oxidation or reduction of the mixed disulfide bond, their presence will influence the amino acid composition of the albumin slightly, and has confused determinations of free α-amino groups. In normal persons about 1% of circulating albumin molecules have incorporated a covalently bound glucose; from this figure the carbohydrate content of "normal" human albumin can be calculated to be 0.0027% (w/w).

The absence of carbohydrate has been used as a criterion of purity of albumin preparations; pure albumin should contain less than 0.05% (w/w) carbohydrate (Hughes, 1954). Absence of carbohydrate and a dearth of tryptophan also mean that acid hydrolyzates of albumin lack the dark precipitate, or humin, found when glycoproteins are hydrolyzed. Albumin itself is colorless. Its affinity for substances such as hematin and bilirubin may cause concentrated solutions to appear yellow.

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TABLE 2-1
Amino Acid Composition of Serum Albumins

Amino acid and parameters	Human[a]	Bovine[b]	Rat[c]	Avg. protein[d]
Aspartic acid	36	40	32	32
Asparagine	17	14	20	24
Threonine	28	34	33	36
Serine	24	28	24	43
Glutamic acid	62	59	57	32
Glutamine	20	20	25	23
Proline	24	28	30	32
Glycine	12	16	17	52
Alanine	62	46	61	49
Valine	41	36	35	38
Cystine/2	35	35	35	19
Methionine	6	4	6	9
Isoleucine	8	14	13	23
Leucine	61	61	56	44
Tyrosine	18	20	21	19
Phenylalanine	31	27	26	22
Lysine	59	59	53	42
Histidine	16	17	15	15
Tryptophan	1	2	1	6
Arginine	24	23	24	25
Total	585	583	584	585
Calc. mol. mass (Da)	66,438.41	66411.17	65,850.51	
Calc. % N	16.5707	16.4698	16.6923	
Avg. residue mass (Da)	113.5704	113.9128	112.7924	
Calc. net charge (pH 7)	215	217	212	

[a]From Fig. 2-1.
[b]From Fig. 2-2.
[c]From Sargent et al. (1981b).
[d]From Doolittle (1987).

The calculated content of nitrogen in the three albumins of Table 2-1 is 16.5–16.7%. Traditionally the nitrogen content of albumins has been assumed to be 16.0% (Watson, 1967); the difference between this and the theoretical values

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must represent small amounts of bound substances, including about 1% tightly bound water and accumulated ligands mentioned just above. The residue after heating, calculated as sulfated ash, is less than 0.05% (w/w), for preparations that have been freed of diffusible salt.

C. Features

With the exception of the cobra, all postteleost albumins of which the sequence is known have 35 half-cystines, which form 17 disulfide bridges (Chapter 4, Section III). Disulfide bonding is a characteristic of extracellular proteins and contributes to the stability of plasma proteins in the circulation.

The most unique structural feature of the albumins is their disulfide bonding pattern. A key to this pattern is the occurrence of eight Cys-Cys sequences involving nearly half of the 17 cystine residues. Although adjacent Cys-Cys pairs *have* been shown to couple to each other (Zhang and Snyder, 1989), they are generally considered as linked to the nearest residues in the chain before and after the pair, to form paired loops 10 to 47 residues long (Fig. 2-1, for example). Whether the bonding pattern is as depicted, with the two loops overlapping at a Cys-Cys pair, or whether the two loops do not overlap at a pair (Fig. 2-3), was not certain. Brown (1975) correctly elected to use the overlapping form in his model; this form is more likely in that (1) it allows the loops to be one residue longer, thus decreasing steric crowding of the smaller loops, and (2) it creates a more rigid backbone at the paired sequences, because rotation about the α-carbon bond between the two half-cysteines is prevented. Foster (1977) noted that a structural model is slightly tighter with the overlapping S–S bonds. This overlapping form of S–S bonds has now been confirmed by the X-ray structure. In actuality, isomerization between the two possible structures may occur, and account for some of the "microheterogeneity" observed for albumin (Section II,C in this chapter and Chapter 7, Section II).

The linear pattern of loops, with only short-range coupling between half-cystines, lends to the albumin molecule both its flexibility and its noted resistance to extreme conditions. The loops can associate to form a globular structure and yet can separate from each other reversibly, under conditions such as acid or strong urea solutions (Section II,C,2).

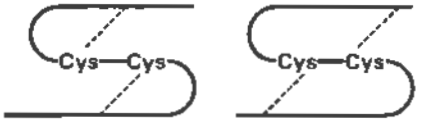


Fig. 2-3. The two possible conformations of disulfide bonds at a Cys-Cys pair, modified from Foster (1960).

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At the amino terminus, loop 1 contains a single loop only, because there is no Cys-Cys pair (at least in species evolutionarily higher than bony fishes—see Chapter 4, Section III). The single free cysteine sulfhydryl is at position 34, approximately the tip of what would be long loop 1 if it existed.

The next obvious characteristic of the primary structure of albumin is the occurrence of a triplet pattern, the nine loops being arranged in the order long–short–long, long–short–long, long–short–long as three homologous *domains*. Homology of sequence among the domains averages 18–25%, and is greatest among long loops 3, 6, and 9. Some features are highly conserved in addition to the disulfide bonding pattern. For all of the eight known mammalian sequences (Chapter 4, Fig. 4-3) the sequence Arg-Arg-His-Pro is at the tip of long loops 3 and 6; it is part of the octa- and nonapeptides (141–150) in human serum albumin (HSA) loop 3, Ile-Ala-Arg-Arg-His-Pro-Tyr-Phe, found to have histamine-releasing (Carraway *et al.*, 1989) and neurotensin (Mogard *et al.*, 1986) activity with mast cells, and resembles the sequence Lys-His-Lys-Pro-Lys-Ala-Thr at the tip of long loop 9. The sequence Lys/Arg-X-Pro likewise occupies the tips of long loops 4, 7, and 8, which is also part of a histamine-releasing peptide, residues 409–423 from the tip of loop 7 (Sugiyama *et al.*, 1989) generated by pepsin, cathepsin D, or macrophage action (Cochrane *et al.*, 1992). Another area of metabolic interest is BSA sequence 152–168, reported to trigger diabetes in infancy by reacting with a B-cell surface receptor (Chapter 6, Section II,B,3,c).

A single Trp residue invariably occurs in long loop 4 in mammals, in a sequence Lys-Ala-Trp-X-Val-Ala-Arg; a second Trp is in a homologous site in long loop 3 in the cow, sheep, and pig. Tyrosine is heavily concentrated in long loops 3 and 6; loop 3 contains five or seven Tyr residues in a stretch only 24 residues long. The nine short loops contain no aromatic residues at all, but tend to be rich in Asp and Glu. Asp is scarce in the long loops. A third of the Lys and Arg residues are adjacent to cystines.

Proline locations are highly conserved. They occur at the tips of all long loops, always with an adjacent basic residue. The amino-terminal half of the molecule contains relatively more glycine, whereas the carboxy-terminal half contains more threonine. The amide forms, Asn and Gln, favor the ends of the molecule over the middle. Other interesting aspects of the distribution of amino acids in the albumin sequence are depicted in Figs. 2-1 and 2-2, and Fig. 4-3 in Chapter 4.

The calculated distribution of net charges shows a gradient along the molecule, domain I having the highest net negative charge; domain II is intermediate and domain III is nearly neutral. The actual net charges may be altered by suppression of ionization of some acidic or basic residues, but the calculated net charge at physiological pH for domains I, II, and III is −9, −8, and +2, respectively, for human albumin, −11, −7, and +1 for bovine albumin, and −10, −5, and +3 for the rat protein. For the two halves of the molecule, defined as

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domain I plus subdomain IIA and subdomain IIB plus domain III, the calculated net charges at pH 7.4 are −14 and −1 for HSA, −12 and −5 for BSA, and −13 and +1 for rat serum albumin (RSA). The dielectric effects of this asymmetry are discussed in Section II.B.2.

D. Fragments

Fragments of albumins have been useful in determining amino acid sequence, disulfide bonding pattern, and the loci of antigenic, ligand-binding, and other functional sites. A distinction should be made between native and denatured fragments; the former are prepared by cleaving the peptide strands between disulfide-bonded loops (Fig. 2-1), whereas the latter are made from albumins following disulfide bond cleavage. Enzymatic (proteolytic) cleavage agents are preferred in preparing fragments, because there is less damage to residues sensitive to oxidation or other modification reactions, but there is still the risk of unwanted cleavages or "nicks" within the fragment. These can be detected by electrophoresis following reduction or by testing for new terminal amino acids. Table 2-2 lists some important fragments that can be prepared by peptide bond cleavage with cyanogen bromide or with limited proteolysis but without cleaving disulfide bonds.

1. Cleavage with Chemical Agents

Chemical agents that will cleave specifically at infrequent amino acid residues are desired. Heating in dilute HCl (0.033 *M*, 105°) releases principally aspartic acid from albumin (Granois, 1960); however, aspartic acid residues are too numerous to make this technique useful. Milder acidity, even 70% formic acid, 30°, 28 h, will specifically cleave the Asp-Pro bond at position 365–366 in albumins of seven species (Votsch *et al.*, 1980). At 5° formic acid does not cause any bond cleavage in 1 week. *N*-Bromosuccinimide under proper conditions cleaves solely at tryptophan residues (Peters, 1959b; Ramachandran and Witkop, 1959). It yields only two or three fragments from mammalian albumins but, because the tryptophans are invariably contained within disulfide loops, the resulting large fragments can be separated only under denaturing conditions (Feldhoff and Peters, 1976).

Cyanogen bromide oxidizes methionine residues, which then cyclize and effect chain cleavage at the resulting homoserine carbonyl groups. As noted in Section I.A, cyanogen bromide fragments are a mainstay of sequencers; Iadarola *et al.* (1988) have published refined separations of the seven cyanogen bromide fragments for use in sequencing human variant forms. Drawbacks with the use of cyanogen bromide are incomplete cleavage at methionines

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TABLE 2-2
Human and Bovine Albumin Fragments Obtained without Cleaving Disulfide Bonds

Loop(s)	Name	Residue numbers[b]		Properties[b]
		Human	Bovine	
(1)	P-Asp[a]	1–24[1]	1–24[2]	Binds Cu(II)[3,4]
1–2	CNBr-B	1–123[5]	—	
1–3	CNBr-N	—	1–184[6–8]	
1–5	P-B[a]	1–308[9, 10]	1–307[11, 14]	
		49–308[15, 16]	—	Binds fatty acid, bilirubin[17–19]
1–6	A	1–380[20]	—	
	P-44	1–387[15, 21]	—	Binds fatty acid, bilirubin,[17, 18] warfarin,[19] B6[22]
		—	1–386[13, 14]	
3	T-42	—	116–185[23, 24]	Binds ANS[17]; insulin stimulation[25]
		141–149[26]	—	Histidine-releasing activity
		181–185[27]	—	Bradykinin activity
3–5	D	116–308[20]	—	
	CNBr-C	124–298[5]	—	
4–5	P-14	—	187–307[14]	Binds bilirubin[17]
4–9	T-23	198–585[28]	199–583[6, 24]	Binds fatty acid,[17, 29] indole,[6] diazepam[29]
	CNBr-C	—	185–583[6–8]	
6	P-9	—	308–386[14, 30]	Induces vesicle fusion
6–9	P-A	309–585[9, 15, 16]	308–583[11, 12, 14]	Binds fatty acid[17]
		(less 408–423[9])	—	
	CNBr-A	299–585[5]	—	
7–9	T-A	—	377–583[23, 24]	Binds fatty acid[17]
		409–423[31]	—	Histidine-releasing activity
8	G	455–480?[20]	454–504[14]	Binds *Streptococcus* protein G
9	P-Phe	495–585[32]	506–583[12, 13, 33]	Contains no Tyr/Phe/Trp

[a]Rat 1–24 (from Ref. 1), 1–308 (from Ref. 10).

[b]Key to references (superscript numbers): (1) Bradshaw and Peters (1979), (2) Peters and Hawn (1967), (3) Peters and Blumenstock (1967), (4) Feldhoff *et al.* (1977), (5) McMenamy *et al.* (1971), (6) King and Spencer (1970), (7) McMenamy and Wesolowski (1972), (8) Goossens *et al.* (1973), (9) Heaney-Kieras and King (1977) (10) Feldhoff and Ledden (1983), (11) Pederson and Foster (1969), (12) King (1973), (13) Braam *et al.* (1974) (14) Feldhoff and Peters (1975), (15) Geisow and Stuchbury (1977), (16) Ledden *et al.* (1982), (17) Reed *et al.* (1975a), (18) Geisow and Beaven (1977), (19) Bos *et al.* (1988c), (20) Falkenberg *et al.* (1992), (21) Bos *et al.* (1988b), (22) Hilak *et al.* (1975), (23) Peters *et al.* (1973), (24) Peters and Feldhoff (1975), (25) Ueno *et al.* (1987), (26) Carraway *et al.* (1989), (27) Weyers *et al.* (1972), (28) Bos *et al.* (1988a), (29) Sjödin *et al.* (1977a), (30) Garcia *et al.* (1984), (31) Sugiyama *et al.* (1989), (32) Bellon and Lapresle (1975), (33) Reed *et al.* (1983).

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(Doyen and Lapresle, 1979), unwanted effects of the formic acid such as the Asp-Pro cleavage cited above, damage to sensitive residues by the acidic and oxidizing conditions employed, and poor solubility of some cyanogen bromide fragments (Khan *et al.*, 1985). Even so, three more or less "native" fragments are readily prepared from human albumin and two from bovine albumin with this technique (Table 2-2).

2. *Cleavage with Proteases*

Early attempts to isolate fragments of albumins utilized chymotrypsin (Porter, 1957; Richard and Kegeles, 1959; Richard *et al.*, 1960). Later, Pederson and Foster (1969) isolated what in retrospect appear from size, terminal residues, and amino acid composition to be bovine fragments 1–307 and 308–583 by limited cleavage with subtilisin. Pepsin and trypsin, however, have proved to be the most useful enzymes.

a. Pepsin. The utility of pepsin in preparing native albumin fragments is the result of (1) its higher selectivity at pH 3–3.7, compared to its normal operating range of pH 1–2, and (2) the expansion of the albumin molecule that occurs between pH 3 and 4, as predicted by Weber and Young (1964b), allowing the pepsin access to the links connecting its loops. Neurath (1986) has discussed the general use of proteases to obtain native domains of multidomain proteins, and Braam *et al.* (1971a) have studied the relationship of pH and the number of peptic fragments obtained.

Following the trail of Weber and Young, Peters and Hawn (1967) isolated two small fragments representing the two ends of the bovine albumin chain (Table 2-2; P-Asp and P-Phe). Braam *et al.* in Nijmegen (1971b, 1974) next obtained fragments apparently corresponding to loops 1–5, 1–6, and 7–9, using 6 *M* urea, and King (1973) prepared the two halves of bovine albumin (loops 1–5 and 6–9) using gentle procedures. This cleavage site in the link between loops 5 and 6 (subdomains IIA and IIB) is in the form of an extended chain in the X-ray-derived structure (Section II,A). Two years later, coincident with the publication of the bovine albumin sequence, a group of peptic fragments was prepared and their locations in the sequence determined (Feldhoff and Peters, 1975), helping to verify the proposed disulfide bonding pattern.

Human albumin has proved harder to fragment. In 1964 Smet *et al.* reported a peptic fragment with antigenic activity, and the amino-terminal peptide 1–24 was obtained (Bradshaw and Peters, 1969), but only in 1977 did Heaney-Kieras and King (1977) achieve the preparation of the two halves of the HSA molecule (loops 1–5 and 6–9), and Geisow and Beavan (1977) produce large fragments containing loops 1–8, 1–7, 1–6, and 6–9. Table 2-2 cites more recent recipes for preparing some of these fragments.

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b. Trypsin. Markus *et al.* (1967b) explored the initial fragmentation of albumin with trypsin, and showed that a pH near 9 favors the formation of larger fragments than does pH 7–8. King and Spencer (1970) applied this powerful enzyme carefully, at pH 8.8 and 0°, to isolate loops 4–9 of bovine albumin. Other tryptic BSA fragments were soon reported using similar conditions (Peters and Feldhoff, 1975; Sjödin *et al.*, 1977a; Bos *et al.*, 1988a), including one constituting domain III (loops 7–9) prepared by digesting BSA heavily with trypsin while it was bound to a palmitoyl–agarose column. Lapresle and his group at the Institut Pasteur in Paris showed following tryptic cleavage that their antigenic "inhibitor" prepared using a heterogeneous spleen cathepsin equaled loop 9 (Bellon and Lapresle, 1975). Serine proteases more specific than trypsin, such as thrombin, have not proved useful in fragmenting albumin owing to absence of the necessary cleavage sites.

3. Properties of Fragments

Isolated native fragments have in every instance validated the proposed disulfide bonding structure. The catalog of fragments prepared (Table 2-2) includes single loops 3, 6, and 9 from both human and bovine albumins, domains I + II from human and bovine albumins, and domains II + III and III alone from bovine albumin. Native fragments have been useful in assessing secondary structure and in locating binding sites for ligands and antibodies, all considered later in this chapter. The fragments possessing disulfide bonds show α helix and β structure by circular dichroism (Reed *et al.*, 1975a); the small P-Asp (1–24) is mainly random coil. Many of the fragments will refold spontaneously when allowed to reoxidize following reduction. Thus the properties of the native fragments support the concept of the albumin molecule as a loose assembly of semiautonomous parts. Binding properties and immunochemical aspects of fragments are discussed in Chapter 3.

Two major fragments will reassemble spontaneously and reversibly at neutral pH. The peptides P-A and P-B, obtained by gentle peptic cleavage of the human 308–309 or bovine 307–308 Asp-Phe bond, dissociate below pH 5, or in 4 *M* urea at neutral pH, but equimolar mixtures will reassociate at pH 7 if the urea is removed (Fig. 2-4). The reassociation can be demonstrated in many ways: by nondenaturing electrophoresis, gel-exclusion chromatography, ultracentrifugation, or osmometry, and by the return of a palmitate-binding locus (Reed *et al.*, 1976; Feldhoff and Ledden, 1983) and of enzymatic activity toward the Meisenheimer complex (Taylor and Vatz, 1973), presumably involving Lys-221 of bovine albumin (Crouch and Kupke, 1980). The same fragments obtained by sub-

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tilisin cleavage (Pederson and Foster, 1969) reassociate between pH 5 and 9 as judged by ultracentrifugation and tyrosyl difference spectral changes at 278 nm.

Feldhoff and Ledden (1983) demonstrated that the association is not species specific. Human and rat fragments 1–308 will complex with bovine fragment 308–583, indicating that the contact points between these fragments have been conserved "in a manner which maintains the overall conformation." The nature of these contact points is considered in Section II,A,4.

The P-A–P-B association has a K_A of 1–2 μM^{-1} (Pederson and Foster, 1969; Crouch and Kupke, 1980), and 94% of a 1:1 mixture of fragments reassociates at pH 8.6 as judged by osmometry. Return of palmitate binding is 74% and of catalytic activity about 73%. The complex also has a larger volume than the parent BSA, largely attributable to swelling of the C-terminal P-A fragment. Hence some nuances of the native molecular structure have been lost in the handling and reassembly.

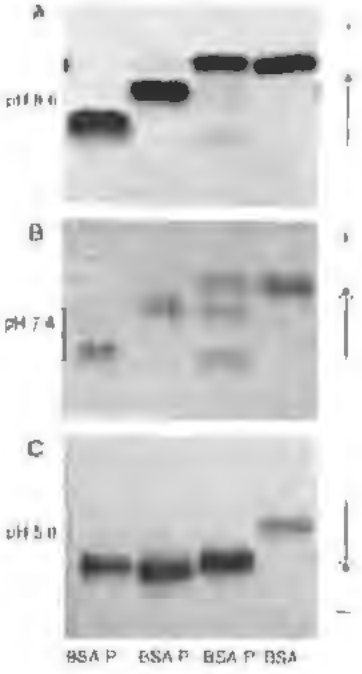


Fig. 2-4. Electrophoresis on cellulose acetate demonstrating the association of two large fragments of BSA to form a molecule similar to BSA. Left to right: BSA 308–583, BSA 1–307, equimolar mixture of the two fragments, intact BSA. (A) pH 8.6; (B) pH 7.4; (C) pH 5.0. The anode is at the top. The fragments are seen to reassociate almost completely at pH 8.6, partially at pH 7.4, and not at all at pH 5.0. Reprinted with permission from Reed *et al.* (1976). Copyright 1976 American Chemical Society.

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II. TERTIARY STRUCTURE AND PHYSICAL CHEMICAL BEHAVIOR

The vast amount of published information about the behavior of albumin in physical chemical studies is impossible to include in a volume of reasonable size. Many of the studies used albumin only as a model protein, and their results might have been gathered with any of a number of other proteins. This section attempts to summarize published data pertinent to obtaining a picture of the size, shape, tertiary structure, flexibility, and accessibility of various residues of human and bovine albumins, and to relate this information to the three-dimensional structure of human albumin recently disclosed from X-ray diffraction. Table 2-3 lists some of the major physicochemical properties of human and bovine albumins.

A. Configuration

1. Molecular Mass

Calculation from the amino acid composition of human, bovine, and rat albumins gives similar molecular masses of 66,438, 66,411, and 65,871 Da, respectively (Table 2-1). The value for BSA compares remarkably well with the figure of 66,700 proposed by Squire *et al.* (1968) in what appears to be the latest critical evaluation of hydrodynamic data (sedimentation equilibrium method). The agreement is even closer when we realize that albumin in solution contains some covalently bound "baggage," such as half-cystine and glycosyl residues, which are not included in the calculation from amino acid composition alone. In the common use of BSA as a calibration standard for molecular mass determination in gel electrophoresis and gel-exclusion methods, a figure of 66,500 Da seems a reasonable recommendation as the best molecular mass available from current data without consideration of bound water.

Published molecular masses for albumins quite early approached the present value. In his 1925 review E. J. Cohn cited a figure of 45,000 Da derived from colloid osmotic pressure measurements (probably on impure or fragmented material). By 1947, however, the Cohn laboratory used a generally accepted weight of 69,000 Da (Cohn *et al.*, 1946). Charlwood (1961) performed extensive ultra centrifugal studies that likewise yielded 69,000 Da for HSA. Measurements of osmotic pressure (Scatchard and Pigliacampi, 1962) showed 68,300 Da for BSA in 0.01 *M* NaCl but 64,700 Da without salt, suggesting the presence of bound (chloride) ions; recent refinements of the method of calculation of osmotic pressure data yielded a mass within 0.9% of the sequence-derived value for BSA (Fullerton *et al.*, 1993; Kanal *et al.*, 1994). Other values from the ultracentrifuge were 65,400 Da (Creeth, 1952) and 64,500 Da (Koenig and Perrings, 1952) for HSA and 66,500 Da for BSA (Baldwin, 1957). From the dimensions of two

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TABLE 2-3
Physicochemical Properties of Human and Bovine Albumins[a]

Property	Human	Bovine
Molecular mass (Da)		
From composition, Table 2-1	66,438	66,411
From hydrodynamic data	65,200[1]	66,700[2]
From ESI-MS	—	65,430[3]
"Best" value in solution	66,500	66,500
Sedimentation constant $s_{20,w} \times 10^{13}$		
Monomer	4.6[4]	4.5[2]
Dimer	6.7	6.7
Diffusion constant $D_{20,w} \times 10^7$, dcm^2/s	6.1[4]	5.9[5]
Translational diffusion constant D_T ($\times 10^{-13}$ m^2 s^{-1})	628	
Partial specific volume V_{20}	0.733[6]	0.733[6]
Intrinsic viscosity, η	0.042[6]	0.041[7]
Overall dimensions, Å		
From physical chemistry	38 × 150[8]	41.6 × 140.9[9]
From crystal structure	30 × 80 × 80[10]	—
Axial ratio	3.9:1[4]	3.4:1[9]
Rotational hydrodynamic radius, Å	26.4[11]	—
Translational hydrodynamic radius, Å	33.1	—
Molecular volume, Å^3, anhydrous:		
Calculated from V_{20}	80,000[12]	81,000[13]
Calculated from crystal structure	88,249[14]	—
Molecular area, Å^2, from crystal structure	28,202[14]	—
Frictional coefficient, f/f^0	1.28[4]	1.30[15]
Isoionic point	5.16[8]	5.15[16]
Isoelectric point (at $\Gamma/2 = 0.15$)	4.7[17]	4.7[17]
Isoelectric point (defatted)	5.8[18]	—
Electrophoretic mobility, pH 8.6, $\Gamma/2 = 0.15$ 10^5 cm^2 v^{-1} s^{-1}	5.9[19]	
Net charge per molecule		
pH 7.4	19[20]	—
Calculated from Table 2-1	15	−17
Refractive index increment (578 nm), $\times 10^{-3}$	1.89[21]	1.909
Optical absorbance, 279 nm, 1 g L^{-1}	0.531[22]	0.667[23]
Mean residue rotation $[m']_{233}$	859[24]	844[25]
Mean residue ellipticity (theta)		
$[\theta]209$ nm	17[26]	21.1[27]
$[\theta]222$ nm	16	20.1
Estimated α helix, %	67[14]	68[28]
β form, %	10	17

[a]Key to references (superscript numbers): (1) Charlwood (1961), (2) Squire *et al.* (1968B), (3) Hirayama *et al.* (1990), (4) Oncley *et al.* (1946), (5) Wagner and Scheraga (1956), (6) Hunter (1966), (7) McMillan (1974), (8) Hughes (1954), (9) Wright and Thompson (1975), (10) He and Carter (1992), (11) Cannistraro and Sacchette (1986), (12) Anderegg *et al.* (1955), (13) Riddiford and Jennings (1966), (14) Carter and Ho (1994), (15) Creeth (1952), (16) Foster (1960), (17) Longsworth and Jacobsen (1949), (18) Gianazza *et al.* (1984), (19) Alberty (1953), (20) Tanford (1950), (21) Armstrong *et al.* (1947), (22) Janatova *et al.* (1968a), (23) Edwards *et al.* (1969), (24) Wallevik (1973b), (25) Moore and Foster (1968), (26) Sjöholm and Ljungstedt (1973), (27) Noel and Hunter (1972), (28) Reed *et al.* (1975a).

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types of crystal structure of HSA, Low (1952) calculated 65,200 and 65,600 Da, with the assumption of a partial specific volume of 0.773. All of the later results are in reasonable agreement with the calculated figure of 66,438 Da; the value of 69,000 Da, based largely on osmotic pressure and light scattering (Edsall *et al.*, 1950), probably overestimated the molecular mass slightly owing to the presence of small amounts of dimers and oligomers, a common concern with methods based on colligative properties. By a more modern technique, electrospray ionization mass spectrometry, Loo *et al.* (1992) found a molecular mass of 66,327 Da for sheep albumin [the value based on amino acid composition (see Chapter 4, Table 4-5) is 66,294 Da]. Cheng *et al.* (1994) found 133,320 Da for the BSA dimer (equal to 66,661 Da for the monomer).

2. *Size and Shape in Solution*

Albumin molecules show birefringence of flow; that is, they tend to align in a rapidly flowing stream like logs in a river (Edsall and Foster, 1948). The viscosity of albumin solutions, although not as high as that of more fibrous proteins such as fibrinogen, is significantly greater than would be the case if its molecule were spherical in shape. Until recently there has been general consensus from these and many other physical measurements that, in solution at least, human and bovine albumins have the cigar-shaped form of an ellipsoid of revolution, with an axial ratio of 3.5:1—a rather stubby cigar to be sure; a closer analogy might be the streamlined hull of an attack submarine (Fig. 2-5).

Two reports using different physical techniques predicted ellipsoidal axes of 40 × 140 Å for BSA in solution, i.e., Squire *et al.* (1968) on reviewing hydrodynamic data and Bendedouch and Chen (1983) based on small-angle neutron scattering. By means of birefringence in an electrical field Wright and Thompson (1975) obtained slightly larger axial dimensions for BSA of 41.6 × 140.9 Å. Studies of the frequency dispersion of the dielectric constant of human (Scheider *et al.*, 1976) and bovine (Rossencu-Motreff *et al.*, 1973) albumins (Section II,B,2) predicted a 3:1 axial ratio. For HSA Oncley *et al.* (1946) predicted dimensions of 38 × 150 Å from hydrodynamic parameters.

It was tempting, considering the triplet homology of the molecule (Figs. 2-1 and 2-2), to regard the three domains within the shell of the hypothetical prolate ellipsoid as aligned linearly like three tennis balls in a can (Fig. 2-5). Bloomfield (1966) proposed such a model from hydrodynamic studies without knowledge of

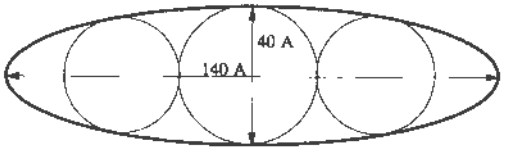


Fig. 2-5. Model of the HSA molecule derived from hydrodynamic studies.

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the homologous domains; he showed three spheres in a row, with diameters of 38, 53, and 38 Å rather than of equal size. The spheres separated from each other by 34 Å when the pH was lowered from 3.6 to 2.2.

The previous year Slayter (1965) actually visualized the albumin molecule as a string of three globules when dried and shadowed by metal in the electron microscope at pH 1.9; at neutral pH no structure could be defined, an implication that the domains separate reversibly under acid conditions. Chatterjee and Chatterjee (1965), however, observed singlet globules of 41 ± 7 × 134 ± 22 Å at pH 8; Höglund and Levin (1965) saw threads on metal shadowing after fixation at pH 6.5, but after fixation at pH 8.8 saw globules about 90 × 65 Å, with an estimated axial ratio slightly over 1.4. They favored a more spherical shape for BSA. Deutsch *et al.* (1962) in the same year reported that some albumin molecules appeared as rings under the ultramicroscope. By the new technique of scanning tunneling microscopy, in 1989 Feng *et al.* found HSA dried on a graphite surface to appear as an elongated (60 × 120 Å) molecule showing three linear domains. Anderson and Weber (1969), using a different approach, polarization of fluorescence of the ligand, 1-anilino-8-naphthosulfonate (ANS), likewise suggested that albumin consists of three segments separated by clefts that provide orientation of the ligand. Foster (1960) explained the expansion of the molecule with increasing acidity with his "club sandwich" model four stacked subunits that dissociate first to two pairs then to four individual plates still linked by a peptide strand. This model fit well with the limited cleavages by pepsin (Section I,D) and subtilisin (Pederson and Foster, 1969), but needed modification when the triplet domain homology was revealed.

X-Ray diffraction now shows that albumin in a crystal, at least fatty acid-free albumin, has a triangular or heartlike shape (see Section II,A,3). This favors the rounded rather than the elongated picture obtained by most physical chemical studies. The different pictures may arise from the flexibility of the albumin molecule, which allows it to assume different configurations when packed tightly in a crystal or floating free in solution, and their reconciliation should be enlightening about its behavior.

Although the peptide chain packs tightly within its shell, be it cylindrical or triangular, there is space for solvent molecules to squeeze in. Drying under severe conditions leaves about 1% residual water detectable by Karl Fischer titration or other techniques (Bull, 1944). The 20 water molecules that Kuntz and Kauzmann (1974) proposed to be bound tightly at specific locations (see Section II,A,1) would correspond to 0.54% of bound water, or an added mass of 360 Da. Infrared spectra of albumin films with increasing hydration show changes in amide I, II, and V bands consistent with binding of this water through hydrogen bonding to oxygen atoms of main-chain peptide carbonyls (Brodersen *et al.*, 1973).

On a larger scale, a monolayer of water molecules, about 33% (w/w), is so closely associated with the surface of the protein by hydrogen bonding as not to

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be freezable. This water layer can be detected by hydrodynamic methods, infrared spectroscopy, dielectric effects in the 200- to 500-MHz range, surface tension calculations from contact angle measurements on concentrated albumin solutions on membranes, or by NMR observations with protons and deuterons (Gallier *et al.*, 1987). The relaxation behavior of BSA after ultrasonic excitation over a frequency range of 0.1–1600 MHz has also been related to the degree of hydration (Choi *et al.*, 1990). Of the associated water molecules, 98% are oriented with their H atoms pointed toward the protein surface, and migrate with the albumin molecule in hydrodynamic studies. The thickness of this layer has been estimated as 5.5 Å (Luzzati *et al.*, 1961), giving a calculated volume for the hydrated molecule of 130,000 Å^3. The next layer of water is only about 30% oriented, and so the slipping plane is between these first and second layers of hydration (van Oss and Good, 1988). This phenomenon creates a "fuzzy" border in solution. It is not unique to albumins, but with their high content of charged and polar amino acid residues it is probably more prominent with albumins than with less hydrophilic proteins. The subject of hydration of proteins has been broadly reviewed by Rupley and Careri (1991).

3. Albumin Structure in Crystals

The three-dimensional structure of serum albumin has been obtained at a resolution of 3.2 Å by He and Carter (1992) at the U.S. National Aeronautic and Space Agency (NASA). This monumental work capped many years of frustration in obtaining usable crystals of albumins, and was aided by crystallization studies under microgravity in United States space shuttles, by greatly improved X-ray beams, and by computerized data analysis.

The first recorded albumin crystals were of horse albumin, hexagonal in shape, which Gürber (1895) and Kekwick (1938) prepared directly from sodium sulfate filtrates of horse plasma; McMeekin (1939) improved on their product in terms of purity. The group under E.J. Cohn prepared many crystalline derivatives from high (40%) ethanol concentrations (Cohn *et al.*, 1947) and studied their properties, particularly those of the mercury (II)–mercaptalbumin dimer (Lewin, 1951). These crystals were discovered by W.L. Hughes when he was investigating the effect of mercuric chloride on albumin, owing to concern over the presence of mercurial preservatives (Cohn, 1948).

Low (1952) obtained monoclinic crystals of HSA, as did McClure and Craven somewhat later (1974), with cell dimensions of 127 × 39 × 135 Å. She also produced orthorhombic crystals from the mercaptalbumin–Hg(II) dimer, of space group $P2_12_12$, and McClure and Craven described a tetragonal form of HSA and a hexagonal form of horse albumin. Rao *et al.* (1976) obtained orthorhombic HSA crystals of cell dimensions 133 × 275 × 58 Å from ammonium sulfate solutions with decanol. Commercial crystalline prepa-

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rations commonly use octanoate as an aid. Low used decanol with HSA, whereas McPherson (1976) added polyethylene glycol to crystallize BSA fragment 308–583. In all of these cases, the crystals were too small for study, or were "soft and waxy" (Low, 1952) or tended to dissolve under study. Water content was about 55%.

Hughes discovered the beneficial effect of decanol on crystallization in a somewhat serendipitous manner (Cohn, 1948). In the days before ultrafilters were available, protein solutions were concentrated *in vacuo* using a capillary stream of air or nitrogen to provide agitation; a few drops of decanol were customarily added to minimize foaming. Hughes found that albumin would crystallize only at lower (e.g., 25%) alcohol concentrations if decanol had been added during the concentration step. A small amount of diethyl ether was also found to be beneficial in lowering the need for decanol.

Carter *et al.* (1989) first reported a structure of HSA to 6.0 Å derived from tetragonal crystals (Fig. 2-6) obtained from defatted commercial albumin. The crystals formed in 0.05 *M* KH_2PO_4, pH 6.8, with the aid of polyethylene glycol of 400 Da. They were of the unusual space group $P42_12$, with dimensions of $a = b = 186.5 \pm 0.5$ Å and $c = 81.0 \pm 0.5$ Å. The crystals had a high solvent content, 78%, with continuous solvent channels of ≈90 Å parallel to the crystallographic c axis.

Despite the high solvent content, the tetragonal crystals were subsequently resolved to 3.2 Å using multiple isomorphous replacement with 12 different derivatives of Pt, Hg, I, and Rh atoms to provide phasing (He and Carter, 1992). A polyalanine model of the complete molecule was constructed using computer graphics, and the known HSA amino acid sequence incorporated into it.

He and Carter also obtained monoclinic crystals from a preparation of recombinant HSA from Delta Biotechnologies, Ltd., of the United Kingdom. These were of the more common space group $P2_1$, with cell constants $a = 58.9$ Å, $b = 38.3$ Å, $c = 60.7$ Å, and $\beta = 101.9°$. They were much drier, with solvent content only ≈33%. The structure was solved to 2.8 Å using molecular replacement technology based on the model derived from the tetragonal HSA crystals. Photographs of a total of eight crystal forms are given in their later review (Carter and Ho, 1994).

Reaching back almost 100 years, the NASA group also used ammonium sulfate, as had Gürber in 1893, and obtained similar hexagonal crystals of horse albumin; they were of space group $P6_1$ with Laue symmetry of $P6/m$ and unit-cell constants of $a = b = 96.6$ Å, $c = 14.3$ Å (Ho *et al.*, 1993); solvent content was 43%. From these crystals a structure of horse albumin at 2.7 Å, determined by molecular replacement with their HSA structure, was found to be very similar to the HSA configuration described below. Structures of dog and turkey albumins refracting to 2.2 and 1.7 Å, respectively, were stated to be under study (Carter and Ho, 1994).

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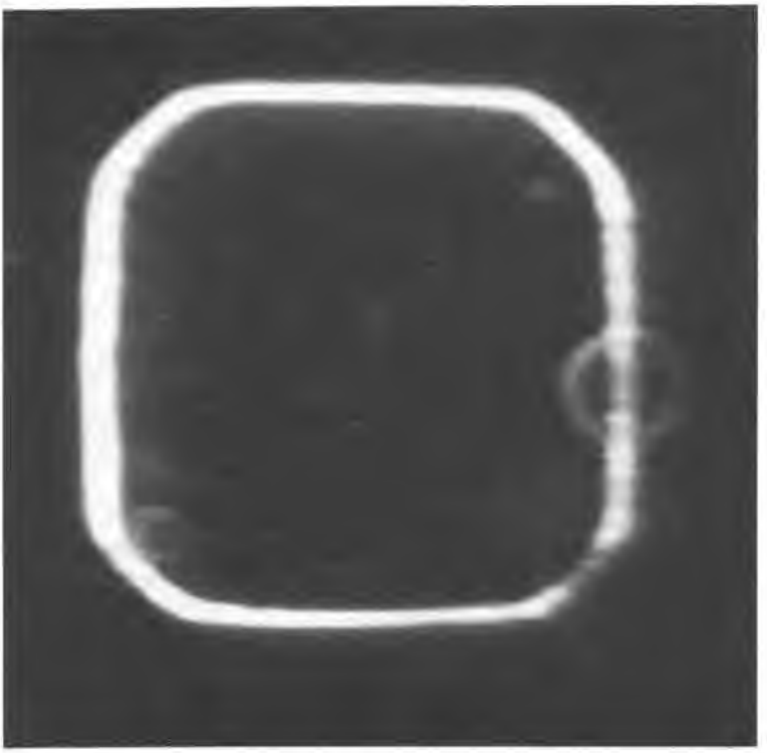


Fig. 2-6. Tetragonal crystal of HSA. Photograph by D.C. Carter of the National Aeronautic and Space Agency, Huntsville, Alabama.

The tertiary structure of HSA derived from these X-ray measurements is a heart-shaped or equilateral triangular molecule 80 Å on a side, with average thickness of 30 Å (Figs. 2-7 and 2-8, colorplates), and a calculated molecular volume of about 88,249 $Å^3$ (Carter and Ho, 1994). Although at variance with the in-line model proposed from hydrodynamic studies (Fig. 2-5), it is supported (1) by the closer agreement of its interresidue distances of Trp^{214}-Try^{411} (19 Å), Trp^{214}-Cys^{34} (33 Å), and Tyr^{411}-Cys^{34} (36 Å) with experimental findings than the linear model would predict, and (2) by its similarity in both size and shape with electron microscopic pictures of dried films of the homologous human α-fetoprotein (AFP) molecule (Luft and Lorscheider, 1983). Note that Morinaga *et al.* (1983) almost simultaneously proposed a triangular structure

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for both AFP and HSA from their calculations of β turns in the predicted secondary structure.

Fluorescent energy transfer predicted the Trp^{214}-Tyr^{411} distance to be 25 Å (Hagag *et al.*, 1983), compared to 19 Å by X-ray. The quenching effect of an azomercurial compound attached to the sulfhydryl group on the fluorescence of the single tryptophan implied the distance between Cys-34 and Trp-214 to be 34–35 Å (Suzukida *et al.*, 1983); Hagag *et al.* (1983) found 31.8 Å, in excellent agreement with the structural distance of ≈33 Å. Fluorescence studies also predicted that Cys-34 lies only 25.2 Å from Tyr-411; X-ray diffraction found a greater distance, ≈36 Å, which He and Carter (1992) conjectured may be due to experimental error and lack of knowledge about the orientation of the fluorescent probe on Tyr-411 and the quencher on Cys-34. Unfortunately, the atomic coordinates of the He and Carter model have not been made public, so that the scientific community cannot make its own distance computations or other assessments.

The general layout of the peptide chain within the crystal structure of Fig. 2-7 is diagramed in one-letter code in Fig. 2-9, which may help the reader in interpreting the structure. The triplet domains are grouped into subdomains A and B. The subdomains are generally tightly packed series of helices; extended peptide strands connect the A and B subdomains, while helical stretches connect adjacent whole domains. The chain folds back on itself at the midpoint, the extended chain between subdomains IIA and IIB, exposing the site for the limited peptic cleavage at residue 307 (Section I.D).

The diagrammatic model of the crystal-derived tertiary structure of Fig. 2-9 is confusing to those of us accustomed to the symmetric linear model of Figs. 2-1 and 2-2, but the relationship between the two models may be more easily understood with the help of Fig. 2-10. In the heart-shaped conformation (Fig. 2-10B), loops 3, 4, 5, and 9 are seen to be inverted, and loops 3, 6, and 9 are displaced out of the linear pattern.

4. *Tertiary Structure in More Detail*

Albumin has long been recognized to be a highly helical molecule, and the X-ray diffraction results show 67% of the residues of crystalline HSA to be involved in a total of 28 α-helical regions. The helical stretches include the residues listed in Table 2-4 and are indicated on Fig. 2-9. The remainder of the chain is extended peptide chain with 10% β turns.

The helical pattern is similar in each of the three domains. Figure 2-11 (colorplate) shows the configuration of domain II as a ribbon model. Subdomain IIA is at the left and subdomain IIB is at the right. A helical segment (h1, h7) starts each long loop (loops 1, 3, 4, 6, 7, and 9), including the half-cystine of the first disulfide bond (except in loop 1, which lacks this residue), but is then broken briefly before

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continuing along the ascending arm (h2, h8). Near the tips of all of the long loops the helix is broken by a short proline-containing sequence. An antiparallel helix runs along the descending arm (h3, h9). It is interrupted in the small S–S-bonded loop but resumes (h4, h10) in an antiparallel fashion in the link to the next small double loop (h4) or domain (h10). Two short antiparallel helices (h5 and h6) occupy the short double loops (loops 2, 5, and 8), and a long strand of extended peptide chain connects these loops to the subsequent long ones and forms the connection between subdomains A and B. Helices h2 and h8 are unique in being the only helices lying entirely within long loops without embracing S–S bonds.

The exposure (and vulnerability) of the long extended strands connecting the subdomains is apparent. In contrast, the connections between whole domains are highly helical. The breaks at the start of the long loops (between h1 and h2 and h7 and h8) form 10-Å openings to pockets formed by the helical segments. These breaks also cause the helices on the descending limbs (h3 and h9) to associate more closely with the following helices (h4, h10) than with the helical segment of the ascending limbs of the same loops (h2, h8).

The helical motifs of the six subdomains within the three domains are related by a pseudo twofold axis, with angles of 168°, 163°, and 171° for domains I–III, respectively (Carter and Ho, 1994). As an indication of the homology between the tertiary structures of subdomains A and B, He and Carter (1992) calculated the root mean square (rms) distances between corresponding α carbons of the superimposed long loops of the three A subdomains (loops 1, 4, 7) and their B subdomains (loops 3, 6, 9); these were 2.47, 2.53, and 2.60 Å for 57 atom pairs of domains I, II, and III, respectively. For entire domains these rms distances were 3.6–4.8 Å.

He and Carter found the helical bundles of subdomains IIA and IIIA to form binding cavities for a number of aromatic small molecules. These two subdomains also have the smallest rms α carbon distance of any of the subdomains, 1.98 Å. The binding regions are discussed later in Chapter 3. The particular amino acid residues that they place in the ligand pockets are listed in Table 2-5 and are indicated in Fig. 2-9.

The albumin tertiary structure is unusual in that each of its adjacent Cys-Cys pairs is included in a helical segment, whereas their bonds always join

Fig. 2-7. Stereoview of bean shaped structure of HSA derived from X-ray crystallography. The locations of the 17 S–S bonds and the side chain of CySH-34 are shown in red. The amino terminus is at the right and the carboxyl terminus is at the left. Reprinted with permission from *Nature* (He and Carter, 1992). Copyright 1992 Macmillan Magazines Limited [The support of Bayer (formerly Miles Laboratories, Inc.) for inclusion of this color figure is gratefully acknowledged.]

Fig. 2-8. Space-filling model of HSA molecule. (A) "Front," (B) "back," (C) left-hand side, (D) right-hand side. As in Fig. 2-7 the amino terminus is at the right in the "front" view. Basic residues are colored blue, acidic residues red, and neutral residues yellow. From Carter and Ho (1994), by permission of Academic Press. [The support of Bayer (formerly Miles Laboratories, Inc.) for inclusion of this color figure is gratefully acknowledged.]

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helices. Only 4 of the 35 half-Cys residues are outside of helices, among them CySH-34. The diffraction-derived structure also confirms, as noted earlier, that the bridging arrangement at the adjacent Cys-Cys pairs is in the overlapping configuration (Fig. 2-3) proposed by Brown. The thioether bonds were found to be *gauche–gauche–gauche*, as predicted for more than half of them by Raman spectroscopy (Aoki *et al.*, 1973). The typical CB_1-S_1-S_2-CB_2 torsion angles are near $\pm 80°$.

Of other amino acid residues that are often classed as helix breakers, 7 of the 24 prolines of HSA, 11 of the 17 asparagines, and 6 of the 12 glycines occur within helices. Sufficient resolution has not been obtained to learn which proline residues are in the *cis* or *trans* configuration; typically in proteins over 90% of prolines are in the *trans* form.

Nearly all of the mutant sites identified so far lie on the surface of the albumin structure (considered in more detail in Chapter 4, Section IV). Similarly, antigenic sites, or epitopes, can be found on the exterior (Chapter 3, Section III). CySH-34, the sole thiol group, and some other active residues are examined in Sections II,B,5 and Chapter 3.

Both fragments P-A and P-B (Section I,D,3) behave on electrophoresis as though they are less negatively charged than intact or reformed bovine albumin (Reed *et al.*, 1976), so some positive charges must be exposed by the cleavage, and these are masked in the parent molecule (Fig. 2-4). This implies that salt bridges are among the forces causing these parts of the albumin molecule to associate. Carter and Ho (1994) indicate these potential salt bridges to be between Lys-190 and Glu-425, Lys-205 and Glu-465, Asp-451 and Arg-218, and Asp-187 and both Lys-432 and Arg-521. However, Crouch and Kupke (1980), from the volume change they found during the association and from an effect of hexane on fragment P-B, predicted that nonpolar interactions predominate. The hydrophobic interactions involve the interdomain cluster Phe-206, Leu-481, Val-482, Trp-214, Leu-347, Val-343, Val-344, Leu-331, Ala-217, and Tyr-452.

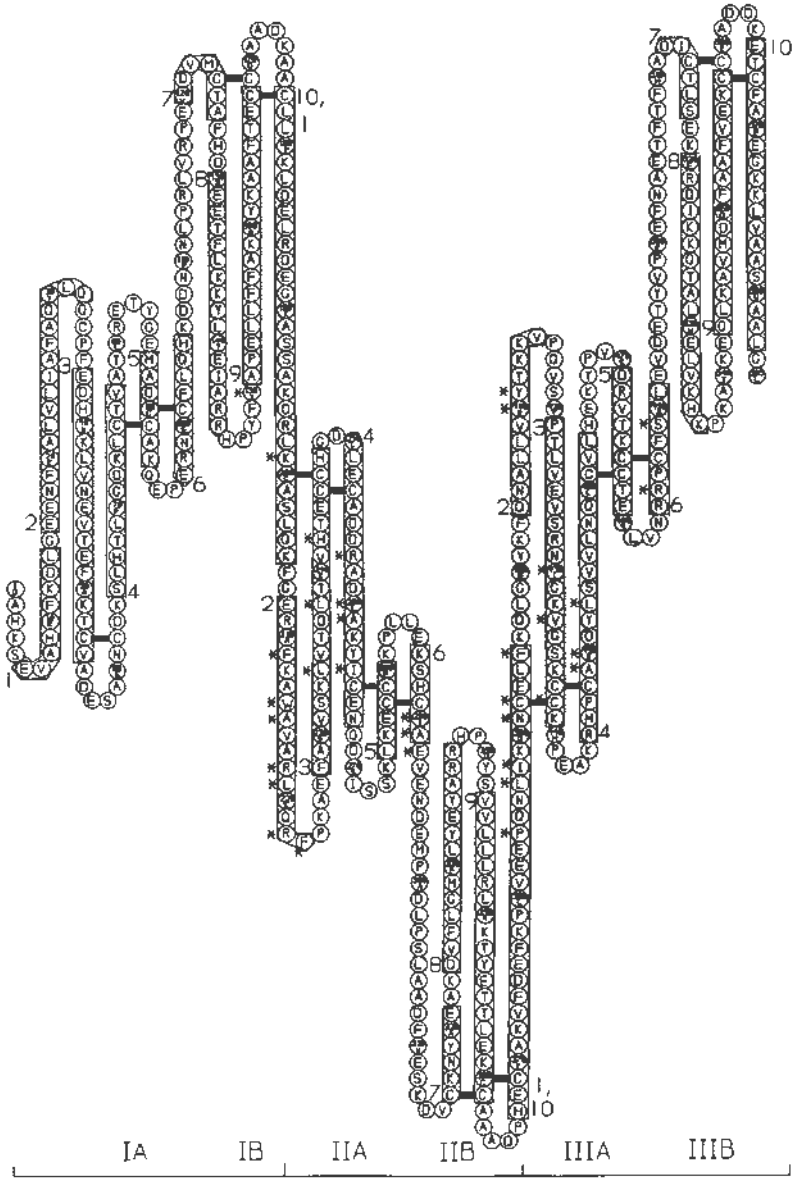
A major question concerning the heart-shaped configuration obtained by diffraction is the role of long-chain fatty acids (LCFAs). To date, results have been published only with crystals obtained from fatty acid-free albumin, yet binding of long-chain fatty acids such as palmitate is known to cause structural changes. With one or two LCFA ligands the carboxyl-terminal portion of the molecule

---

Fig. 2-11. Ribbon model of domain II of HSA. The disulfides are shown in red. The ten principal helices (Table 2-4) are labeled h1 to h10. Reprinted with permission of *Nature* (He and Carter, 1992). Copyright 1992 Macmillan Magazines Limited [The support of Bayer (formerly Miles Laboratories, Inc.) for inclusion of this color figure is gratefully acknowledged.]

Fig. 2-13. Stereo ball–stick model of the structure around residue CySH-34 of HSA. Red, oxygen; yellow, carbon; blue, nitrogen; green, sulfur. From Carter and Ho (1994) with permission of Academic Press. [The support of Bayer (formerly Miles Laboratories, Inc.) for inclusion of this color figure is gratefully acknowledged.]

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apparently becomes more compact, the molecule resists proteolytic degradation and heat denaturation, the dielectric constant falls, and bilirubin binding is enhanced. It remains to be seen whether LCFA-induced conformational changes affect the configuration seen in albumin crystals, and whether the structure in a crystal is the same as that in solution.

5. *Predictions of Helical Structure*

The presence of α helix, β sheets and bends, and random coils can be predicted by several physical and sequence-related methods, and it is instructive to compare, in retrospect, the results of the various methods to see which agree best with the 67% helix, no β sheet, 10% β turn, and 23% extended chain found by X-ray crystallography for the human albumin molecule. Most of these are based on comparisons with X-ray diffraction-derived structures of other proteins, however, and so are inherently less accurate than the X-ray structure.

The earliest optical measurements in solution were made with BSA, probably owing to availability of material purer than HSA. Optical rotatory dispersion (ORD) and circular dichroism (CD) studies predicted an α helix content ranging from 54 to 68% (Sogami and Foster, 1968; Reed *et al.*, 1975a). Raman spectroscopy (Chen and Lord, 1976) found 60%, based on analysis of the amide III lines. For HSA, ORD and CD forecast 44 and 48% helix, respectively (Jacobsen, 1972; Sjöholm and Ljungstedt, 1973).

Numerous computerized programs have been devised to predict secondary structure by comparison of amino acid sequences of a protein with those of a collection of proteins with published configurations. The older program of Chou and Fasman (1974) predicted a helical content of only 48% compared to the 67% found by X-ray. Its predictive accuracy for the location of helical regions was only 33%. A recent refinement, the "PHD" method (Rost and Sander, 1993), predicted 59% helix, with 79% agreement with the locations from X-ray. The sequence-based program of Pearson (Pearson, 1990) predicted α helix for HSA of 65%, with 10% β sheet and 19% β turn.

J.R. Brown constructed a space-filling model from the sequence of domain II of BSA (Brown and Shockley, 1982), based on his proposal that each domain contains six helices, each containing six turns (about 19 amino acid residues).

Fig. 2-9. Amino acid sequence of HSA in an arrangement reflecting the heart-shaped structure (Fig. 2-7) based on a design by D.C. Carter of NASA. Approximate helical regions (Table 2-4) are boxed and numbered at their starting points, numbered 1–10 for each domain. Ligand sites in subdomains IIA and IIIA (Table 2-5) are indicated by asterisks. The calculated net charge by quadrants for this configuration is as follows: upper left, −8; upper right, +2; lower left, −6; lower right, −3. The one-letter abbreviations for amino acids are A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; Y, Tyr.

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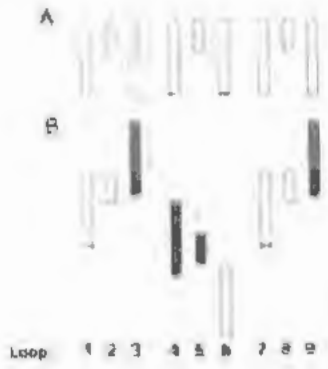


Fig. 2-10. Relationship of the X-ray heart-shaped structure (B) to the linear model (A). Loops in an inverted orientation are filled. Note that loops 3, 4, 5, and 9 are inverted and the long loops 3, 6, and 9 are displaced from the linear formation.

Within a domain the six helices are oriented in an *antiparallel* manner. The inner faces of the helices create a hydrophobic surface within each domain; this forms a channel that could accommodate the tail of a long-chain fatty acid such as palmitate. Although somewhat of a simplification of the X-ray findings, his proposal and his model are in large measure accurate.

TABLE 2-4
Approximate Locations of α Helices in HSA[a]

	Domain I		Domain II		Domain III	
Name	Residues	Size	Residues	Size	Residues	Size
	IA		**IIA**		**IIIA**	
h1	6–14	9	(177–205)	(29)	(367–395)	(29)
h2	16–32	17	208–223	16	404–415	12
h3	37–54	18	228–247	20	421–439	19
h4	65–77	13	250–267	18	445–463	19
h5	87–93	7	275–280	6	471–479	9
h6	97–105	9	286–291	6	484–491	8
	IB		**IIB**		**IIIB**	
h7	120–126	7	316–321	6	512–517	6
h8	130–145	16	324–337	14	520–536	17
h9	151–168	18	343–361	19	543–558	16
h10	177–205	29	367–395	29	565–583	19

[a]From Carter and Ho (1994).

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TABLE 2-5
Amino Acids Involved in Ligand Sites[a]

Sudlow site I: Subdomain IIA		Sudlow site II: Subdomain IIIA	
Helix	Residue	Helix	Residue
None	Tyr-150??	h1	Pro-384
h1	Lys-199		Leu-387
h2	Phe-211[b]		Ile-388
	Trp-214		Asn-391
	Ala-215[b]		Cys 392[b]
	Arg-218[c]		Phe-395[b]
	Leu-219	h2	Arg-410
	Arg-222		Tyr-411
	Phe-223	h3	Leu-430
h3	Leu-234		Val-433
	Leu-238		Cys 438[b]
	His 242	h4	Ala-449
h4	Arg-257		Glu-450
	Leu-260[b]		Leu-453
	Ala-261[b]	h6	Arg-485
	Ile-264		Ser-489
h6	Ile-290		
	Ala-291		
None	Glu-292[b]		

[a]From Carter and Ho (1944)
[b]More remote but still form important sides of the binding site.
[c]Still farther away but involved.

6. *Flexibility*

The albumin molecule is not in a static, "platonic" state, but has been described by Weber (1975) as flexible and rapidly changing in shape, a "kicking and screaming, stochastic" molecule. The whole molecule tumbles in about 40 nsec (rotational diffusion coefficient). Its loop-link structure permits rapid expansion, contraction, and flexion, some of it intrinsic, some of it related to binding of ligands. The concerted motions of the albumin molecule to accommodate ligand binding (q.v.) occur in 0.1–0.3 s. Choi *et al.* in 1990 applied the technique of

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ultrasonic spectroscopy and found that expansion and helix–coil transitions relate to pressure wave absorptions at 200 kHz, corresponding to times of 5 μsec for these transformations. Proton transfers of carboxyl and phenolic groups resonated at 500 nsec, and of amino groups at 20 nsec. Molecular oxygen, a quencher of tryptophan fluorescence, can enter the 10-Å crevice between helices h1 and h2 (Fig. 2-11A), beneath which lies the sole tryptophan of human albumin, within 6 nsec (Gurd and Rothgeb, 1979).

The rate of exchange of potentially labile hydrogen atoms with hydrogens of the surrounding water is frequently applied to measure the flexibility and speed of motion of protein molecules. The exchange is measured either by isotope replacement with tritium, using its radioactivity as a label, or, more commonly, with deuterium, which can be measured by specific gravity in the Cartesian diver, by infrared spectroscopy, or by nuclear magnetic resonance (Hvidt and Wallevik, 1972). At physiological pH, about 750 of 1100 potentially exchangeable hydrogens exchange too rapidly to be detected with this technique, even at 0°. Another 280 exchange in two classes with first-order rate constants of about 10^{-3} and 10^{-5} s^{-1}; the remaining 70 fail to exchange after 24 h at 25° (Hvidt and Wallevik, 1972). Benson and Hallaway (1970) propose that much of the motility of bovine albumin is the result of independent segmental movements of parts of the molecule; these can be equated with the subdomains of Fig. 2-7.

High exchangeability of hydrogens is a unique characteristic of albumin among nonenzymatic proteins (Willumsen, 1971), and is probably a corollary of its loose structure and its propensity for binding many ligands. Ovalbumin and β-lactoglobulin, for example, exchange deuterium atoms considerably less rapidly. Studies with tritium showed that HSA has an average accessible hydrophobic area of 130 $Å^2$ compared to only 10 $Å^2$ for lysozyme (Volynskaia *et al.*, 1985).

Like other proteins, components of albumin are also constantly moving on more rapid time scales. Gurd and Rothgeb (1979) have reviewed these motions. Reorientation of amino acid side chains occurs in 10^{-10} 10^{-11} s, and ionizations in 10^{-11} s. Amino acid side chains not restricted by π bonding rotate about their single bonds; a methyl group rotates in 5×10^{-12} s. Even the single tryptophan side chain of human albumin, observed by time-resolved fluorescence spectroscopy (Munro *et al.*, 1979), rotates independently at a rapid rate, about 10^{-10} s rotational correlation time.

Hence, although albumin in solution can be considered as having a single shape overall, it is probably more realistic to regard it as an assembly of squirmy, resilient parts, frequently changing in conformation through opening and closing of major crevices. With this "breathing" action, and with many of its amino acid side chains constantly in motion on a micro scale, the albumin molecule is well fitted to assimilate or release the many substances that it transports in the circulation.

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B. Physical Chemistry

In this section we will consider the physicochemical properties of human and bovine serum albumins, especially as they can furnish information on the behavior of various chemical groups within the albumin molecule in relation to the tertiary structure. Physicochemical effects related to molecular isomerization, ligand-binding sites, and antigenic epitopes are not discussed here, but in Section II, C and Chapter 3.

1. Electromagnetic Spectral Properties

Electromagnetic spectral techniques detect chiefly chemical groups exhibiting radiative activity: absorbance, fluorescence, polarization, rotation of light, and Raman radiation. The responsible groups are principally the aromatic amino acids, tryptophan, tyrosine, and phenylalanine, listed in order of activity. Human albumin has but one tryptophan, at position 214 in loop 4; bovine albumin has a homologous tryptophan, at position 213, plus a second one in loop 3 at position 134 (Figs. 2-1 and 2-2). As noted earlier, the 18 tyrosines of human albumin are concentrated in loops 3 and 6 (five residues each); the 20 tyrosines of bovine albumin are found in entirely homologous locations, with the additional two residues being found in loop 3 (giving it a total of seven tyrosine residues). Note that loop 4, containing the common tryptophan, has no tyrosines, whereas loop 3 of BSA, containing the second tryptophan, has seven tyrosines. This difference in vicinal aromaticity may contribute to the difference in properties of the two BSA tryptophans.

a. Absorbance. In the visual spectral range (400–800 nm) solutions of pure albumin are colorless—they absorb no light. If there is residual yellow color it is usually the result of small amounts of retained bilirubin, carotene, or, particularly with commercially produced HSA, hematin or degradative products of *N*-acetyltryptophan.

In the *near-ultraviolet* range (240–400 nm) the absorbance spectra of albumins are similar to those of most simple proteins (proteins without prosthetic groups), with a peak near 280 nm (Fig. 2-12a). Owing to the paucity of tryptophan residues, however, their absorptivities are atypically low, 0.5–0.7 (Table 2-3) compared to about 1.0 for most proteins. This reflects the greater influence of tryptophan compared to the other aromatic amino acids; the molar absorptivities for tryptophan, tyrosine, and phenylalanine, $\epsilon_{280\ nm}$, are 5540, 1480, and ≈ 0, respectively.

The molar absorptivity peak for HSA near 280 nm is about 20% lower than that for BSA (Fig. 2-12a), because it has but a single typtophan compared to two for BSA. The absorptive peaks for HSA and BSA are not exactly at 280 nm, the

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wavelength commonly selected for measurement of proteins in solution, but at 278.5–279 nm. The difference between the absorbance of BSA at 278.5 and 280 nm is about 1%, 0.667 versus 0.661. There is little change in the absorbance near 280 nm between pH 5 and 8, and it is unaffected by ionic strength between 0 and 0.3 *M*.

Calculation of the $A_{280\ nm}$ for albumins from their composition, including a contribution from S–S bonds ($\epsilon_{280\ nm}$ = 134), gives reasonable agreement with the measured values. For HSA, BSA, and RSA the calculated absorptivities are 0.52, 0.65, and 0.55, respectively (Mach *et al.*, 1992), compared to 0.531, 0.661 (Table 2-3), and 0.59 (Peters, 1985). Similar calculations give reasonable absorptivities for albumin fragments and cloned domains as well.

In the *far-ultraviolet* region, below 240 nm, the absorbance of the peptide bond predominates, peaking near 187 nm (Rosenheck and Doty, 1961). The

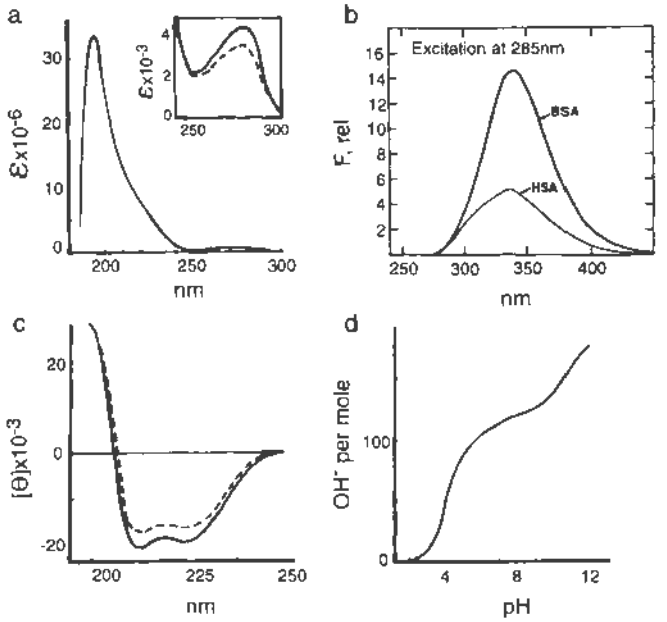


Fig. 2-12. Some physical chemical properties of albumins. (a) Molar absorptivity; (b) fluorescence emission (relative); (c) mean residue ellipticity by CD; (d) acid–base titration curve. Solid lines, BSA; dashed lines, HSA. The titration curves of HSA and BSA do not differ significantly. Modified from Peters (1975) and Steinhardt *et al.* (1971), with permission of the publishers. (b) Reprinted with permission from Steinhardt *et al.* (1971). Copyright 1971 American Chemical Society.

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absorbance is very high but is strongly affected by traces of turbidity or the presence of carboxyl or hydroxyl ions, and its measurement places severe requirements on the optics of the spectrophotometer and the purity of the albumin solutions. $A_{1\ g/L}$ at 190 nm for BSA is about 50 times the peak absorbance at 280 nm.

The minimum between the 187- and 280-nm peaks occurs near 253 nm. It, too, is affected by traces of turbidity and other impurities; according to the author's training, a good preparation of albumin shows a minimum absorbance $\leq 0.5 \times$ the absorbance maximum at 280 nm.

Difference readings at 287 nm, where tyrosine absorbance is strongest, offer predictions on the relative intramolecular location of tyrosine residues. About two-thirds of the 18 tyrosines of human albumin are accessible to solvent effects (Steinhardt and Stocker, 1973). Of these, about six appear to lie near enough to the surface of the protein to be perturbed by bulky solvent molecules such as polyethylene glycol (Herskovits and Laskowski, 1962). One of these six was placed near Cys-34, based on the effect of blocking of the single sulfhydryl group.

About four tyrosines of BSA show increased absorption at pH 3.8, when the albumin molecule expands to its F form (Section II,C,1,a), indicating exposure of some internal hydrophobic surfaces to the solvent environment. Another four tyrosines become exposed when the pH is dropped below 3.5 in the presence of organic solvents (Glazer *et al.*, 1957; Williams and Foster, 1959).

The six most sequestered tyrosines of either HSA or BSA require major alteration of the albumin structure for optical activity—reduction of the 17 disulfide bonds plus the presence of 8 *M* urea (Herskovits and Sorensen, 1968). The conclusions from absorbance spectral changes in the near ultraviolet range are that one-third of the 18–20 tyrosine phenols are readily accessible on the molecular surface, another third are accessible on pH changes that cause globular parts of the molecule to separate, and the final third are exposed only on strenuous, irreversible opening of the structure (Section II,C,2).

D.C. Carter, at the author's request, made a "cursory look" at the tyrosine locations with these predictions in mind (Carter, 1994). He used the horse albumin structure because it was known with higher resolution than the human. By homology it appears that five obviously exposed tyrosines in the human structure are residues 148, 263, 332, 401, and possibly 497. Seven partially buried tyrosines are 84, 138, 140, 161, 319, 334, and 341, and six obviously buried tyrosines are 30, 150, 353, 370, 411, and 452. Tyr-30 and Tyr-84 are the ones closest to CySH-34 (see Section II,B,5); neither of these was found to be on the surface of the molecule as predicted by Herskovits and Laskowski (1962).

b. Fluorescence. Fluorescence, the immediate emission of light on radiation with monochromatic light, is largely attributed to the tryptophans of albumins.

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There is only a minor contribution by the more numerous tyrosines, and that is dependent on the wavelength of the exciting light. When this wavelength is between 295 and 305 nm, tyrosines are not excited, and only a pure tryptophan emission spectrum, centered near 345 nm, is seen. The precise λ_{max} is determined by the environment of the tryptophan (Steinhardt *et al.*, 1971).

Most studies are concerned with the tryptophan fluorescence, and use excitation at ≥296 nm. If desired, the contribution of tyrosines can be estimated with excitation below 295 nm, when both aromatic side chains are excited, subtracting the tryptophan contribution to emission excited at ≥296 nm.

Figure 2-12b shows the spectra of the emission from HSA and BSA excited at 285 nm. The maximum energy from BSA is about 2.7 times that from HSA. Steinhardt *et al.* (1971) concluded from this that the single tryptophan of HSA is partially quenched, whereas both of the tryptophans of BSA are essentially unquenched. Indeed, BSA emits fluorescence with an intensity very nearly the same as that given by two molecules of the free indole, *N*-acetyltryptophanamide. With excitation at 285 nm, some (about 20%) of the energy from excited tyrosines of HSA can transfer nonradiatively and act as inciting energy to the nearby tryptophan. This effect is smaller for BSA, in which the emission from tyrosines is largely quenched. Like absorption of light, the fluorescence of HSA is fairly constant from pH 5 to 9.

Because the single tryptophan of HSA lies in a position similar to that of one of the tryptophans of BSA (Trp-214, loop 4—see Figs. 2-1, 2-2, and 2-9), it has been tempting to assume that the fluorescence of the tryptophan of HSA corresponds to that from the BSA tryptophan in loop 4, that *differences* between the fluorescence of BSA and HSA are the result of the unique tryptophan of BSA, Trp-134 in loop 3.

The *commonly situated Trp-214* lies in a conserved sequence on the ascending limb of loop 4. The concept prevalent among investigators is that it is buried and protected from exposure to polar solvents—it lies in a "very flabby hydrophobic protein matrix," to quote Eftink and Ghiron (1977). The bulky iodide ion, however, can gain access and strongly quench the fluorescence of HSA (Noel and Hunter, 1972). The even larger octanoate molecule approaches within 10 Å and increases the quantum yield of fluorescence (Steinhardt *et al.*, 1971). Quenching of HSA fluorescence by *N*-bromosuccinimide (Peterman and Laidler, 1980) is biphasic; it involves a second-order reaction, perhaps indicating attachment at the mouth of a hydrophobic fold, followed by a first-order conformational change permitting access of the quencher to the tryptophan.

Not all studies agree that the common tryptophan of HSA and BSA is buried in the molecule. Photooxidation of the tryptophyl residue can readily be sensitized by bulky dyes such as methylene blue or rose bengal—as readily as the sensitization of a free tryptophan compound. This prompted Reddi *et al.* (1987) to predict that the single tryptophan of HSA is situated near the surface

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of the molecule. Conflicting results have also resulted from the technique of optically detected magnetic resonance (see Section II.B.1.c).

X-Ray diffraction places Trp-214 of HSA within the binding pocket of subdomain IIA, near the start of helix h2 of domain II. In the review by Carter and Ho (1994), Trp-214 can be visualized as lying on the left side of this pocket. The access opening to this pocket is about 10 Å wide; the presence of the tryptophan and two guardian tyrosines purportedly limits accessibility of solvent to the pocket. This location substantiates the buried rather than a surface site for this tryptophan.

The *unique Trp-134* of BSA is in a less strongly conserved but homologous region on the ascending limb of loop 3. Although there are conflicting interpretations, most physical studies predict that the second tryptophan of BSA is nearer the molecular surface than the HSA tryptophan, but not *on* the surface. Feldman *et al.* (1975) concluded from the quenching effect of glycerol and Cu(II) ions that one of the BSA tryptophans is appreciably nearer the surface than the other. Octanoate quenches the fluorescence, and the quenching by *N*-bromosuccinimide is first order (one stage) (Peterman and Laidler, 1980). As the pH rises from 8 to 9, there is apparently quenching by ϵ-amino groups that become deprotonated unless chloride ion is present (Halfman and Nishida, 1971b).

The immediate environment of this second tryptophan appears to be more constrained than the first. The polarization of its fluorescence indicates restriction from rotation (Sogami *et al.*, 1975), perhaps by nearby tyrosyl groups. Iodide ion cannot normally gain access for quenching (Noel and Hunter, 1972), but can when the molecule is spread into a foam (Clark *et al.*, 1988).

The second tryptophan of BSA by analogy to the X-ray structure of HSA would lie in helix h8 of loop 3 in subdomain IB (Fig. 2-9). This site is not at the surface, nor is it within as well-defined a binding pocket as is Trp-214. The apparent constraint of the residue may indeed arise from the nearby tyrosyl groups, which are more numerous in BSA than in HSA.

c. Phosphorescence and Optically Detected Magnetic Resonance. Optically detected magnetic resonance (ODMR) was apparently first applied to albumin in 1982 (Bell and Brenner, 1982). In this procedure the excited triplet state of tryptophan, as a chromophore, is used as a spin probe; its magnetic resonance transitions are detected by optical methods. The phosphorescence at 77°, ODMR line width, and zero-field splitting frequency all indicate that the single tryptophan of HSA is buried in a hydrophobic environment, in essential agreement with the fluorescence studies. As with fluorescence, iodide ion exhibited a heavy-atom quenching effect with HSA. Hence this technique also agrees with the location of Trp-214 determined by X-ray diffraction.

ODMR results with BSA and its cyanogen bromide fragments 1–184 and 185–583 (Mao and Maki, 1987), each containing one of the two tryptophans, were essentially additive to yield the results with intact BSA. These authors'

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conclusions, however, differed from those of Bell and Brenner (1982) and from the findings with fluorescence by placing the common tryptophan (Trp-213 of BSA) in an only partially buried environment, with inhomogeneity suggesting exposure to solvent. The second tryptophan of BSA, on the other hand, was predicted to lie interiorly in a hydrophobic environment.

d. Raman Spectra. Raman spectra, the series of frequency-shifted emissions following laser excitation, have been investigated with BSA. About half of the bands could be attributed to the rings of the three aromatic amino acid species and to S–S and C–S bonds (Bellocq *et al.*, 1972; Lin and Koenig, 1976). Using $\pi \rightarrow \pi^*$ and resonance Raman measurements, Chen and Lord (1976) also identified C–O, C–C, and C–N bonds. Overall, however, this technique has not yet yielded detailed structural information.

e. Nuclear Magnetic Resonance. Many of the early NMR studies on albumins dealt with the protons of albumin in solution (Aksenov and Kharchuk, 1975; Grösch and Noack, 1976). One study observed the hydrogen atoms of water as albumin was rehydrated from a powder form (Blears and Danyluk, 1968). In solution three classes of proton behavior were seen: protons in bulk water, protons in water loosely bound in a monomolecular layer around the albumin molecule, and protons of the albumin. The tumbling of the macromolecule could be observed in one of the relaxation times, as well as a contribution at a lower frequency from segmental motions (Gallier *et al.*, 1987). The albumin protons observed are primarily the acidic ones that can exchange with hydrogen or deuterium atoms of the surrounding water.

Among specific amino acid constituents, Bradbury and Norton (1973) have reported the ^{13}C NMR spectra of albumin tryptophans, and Sadler and Tucker (1992) have assigned resonances to the first three N-terminal residues of human, bovine, rat, and pig albumins, presuming that the N terminus would be the most flexible region of the molecule. This appeared to be the case on study of albumin crystals; no electron densities were resolvable for these amino-terminal residues. They propose a pK_a for the NH_2 of Asp-1 of 7.8. Possible peaks for Lys-4 and Ser-5 were also noted, but none for Glu-6, suggesting that mobility of the N terminus is already restricted at residue 6. For BSA, side chains of Thr-190, Tyr-156, His-59, and His-378 were assigned to peaks. Contaminating glycoproteins were identified by their *N*-acetyl resonances, and disulfide-bound half-cystine was detected on reduction with thiols.

The C-2 protons of histidines are often discernible with NMR. Silber (1974) reported the effects of ionization between pH 6 and 8, whereas Bos and co-workers (Labro and Janssen, 1986; Bos *et al.*, 1989b) have begun to assign 17 resonances and their behavior on titration between pH 5 and pH 9 to particular HSA histidines, aided by studies on fragments 1–384 and 198–585. The imidazole pK values ranged between 5.5 and 8. The C-2 H of His-3, which

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creates a copper-binding site (Chapter 3, Section II.A.1), was readily identified, as well as that of His-464. His-464 can be seen to lie just outside of a helical stretch, helix h5 of domain III. Other resonances could be assigned to particular fragments, but we see that only two of the resonances of the 17 histidines of HSA have as yet been identified. NMR has also helped to locate ligand sites (Chapter 3) and histidine residues involved in the **N** → **B** isomerization (Section II.C.1.c).

f. Other Spectral Techniques. Optical rotatory dispersion and its related technique, circular dichroism, were discussed in Section II.A. The CD pattern (Fig. 1-12c) is typical of highly helical proteins. The curve for BSA is more pronounced than the one for HSA, in keeping with the somewhat higher estimates of helical content in BSA.

Infrared spectroscopy has mainly been applied to the detection of water molecules (Brodersen *et al.*, 1973) and, by the ratio of amide I to amide II lines, to the assessment of helical content (Kato *et al.*, 1987). Vibrational circular dichroism of BSA in the amide I' region has been observed. It shows mainly short-range interactions and complements more established techniques (Pancoska *et al.*, 1991). Measurements of the angular dependencies of the Rayleigh scattering of Mössbauer radiation have been reported from Russia (Krupianskii *et al.*, 1992); they were interpreted in terms of motions of side chains and whole helical regions. Electron spin resonance is employed mainly with reporter compounds in the study of ligand sites.

2. *Ionic Properties*

The *isoionic point* of albumins, the pH of a thoroughly deionized solution, is about pH 5.2 (Table 2-3). At this pH essentially all of the carboxylic acids are deprotonated and the amino, guanidino, and imidazole groups are protonated, so it is also the pH of maximum calculated total charge, about 100 each positive and negative charges. By definition there are no adherent charges such as salt ions. The exact total charge is not known; as we have seen, some carboxyl groups may be buried and un-ionized (i.e., protonated), perhaps bonding with nonprotonated imidazole or ε-amino groups.

In the presence of increasing concentrations of salts such as NaCl, bound ions influence the charge on the albumin molecule. G. Scatchard studied the binding of small anions to albumin in detail between 1944 and 1964 (paper XII in his series is Scatchard *et al.*, 1964). His work showed the effect of increasing sodium chloride concentration on the binding of chloride ion to be calculable as:

$$Cl^-/\text{albumin (mol/mol)} = 13.5 + 5.6 \log[Cl^-] \text{ (in mol/kg } H_2O). \quad (1)$$

The introduction of ion-specific electrodes, which measure only unbound ions, made more precise determinations possible. At pH 7.4, in serum or equivalent salt

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solution, seven to eight chlorides bind per albumin molecule (Fogh-Andersen *et al.*, 1993); NMR with ^{35}Cl indicated 10 or less (Halle and Lindman, 1978). Location and strength of binding are discussed later (Chapter 3, Section I,D,4, and Table 3-1). As the pH is lowered, chloride binding increases, to 11 ions/molecule at pH 5.2 and 22 at pH 4.2. Monovalent cations, sodium and potassium, are bound significantly only above pH 7.4.

The *isoelectric point*, in contrast to the *isoionic point*, is the pH at which the net charge of a molecule, including any bound ions, is zero. This is the pH at which a protein will not migrate in an electric field, as well as the pH zone in an isoelectric focusing gradient to which it will move and remain stationary. For undefatted albumin in 0.15 *M* NaCl the isoelectric pH is about 4.7 (Table 2-3); bound chloride and fatty acid ions cause it to be lower than the isoionic point.

At pH 7.4, the pH of blood, the net charge on the albumin molecule calculated from its amino acid composition is −15, −17, and −12 for HSA, BSA, and RSA, respectively (Table 2-1). This is also the relative order of anodal migration of these albumin species on electrophoresis at pH 7–9.

For HSA at pH 7.4, adding −7 for bound chloride ions, the net molecular charge becomes −22; with 42 g/L (0.64 m*M*) of albumin in plasma, the charge contributed by albumin is −14.1 mEq/L. (Bound fatty acid may raise this figure but bound calcium would lower it; see Chapter 3, Sections I,A and II,B). This is actually a little larger than the net charge of −12 on the total protein of plasma measured by Figge *et al.* (1991). These authors derived a formula for calculating the pH of plasma from the pO_2, the net strong ion (salt) charges, the inorganic phosphate concentration, and the albumin concentration. They concluded that albumin alone is significant as a net negative protein ion in plasma, accounting for the bulk of the clinically unmeasured anions. (The other normally unmeasured anions are carboxylates such as lactate and citrate.)

The titration curve of a protein is the composite curve of its many amino acid ionizable groups. The titration curve of albumin (Fig. 2-12d) shows several unusual features. For much of the information about the titration of albumins we are indebted to Tanford (1950), Steinhardt *et al.* (1971), and the review by Foster (1960), to which the reader interested in the development of equations from Debye–Hückel theory is referred.

The titration curve is flattest between pH 5 and pH 8, so that albumin is a rather weak buffer in the physiological pH range. Here it is mainly the imidazoles of the histidines and the terminal amino and carboxyl groups that are being protonated. The net charge is also affected slightly in this range by calcium binding. Figge *et al.* (1991) derived the HSA titration curve in the pH range 6.6 to 8.2 mathematically using the actual p*K* values for the 16 histidine imidazoles obtained from ^1H NMR (Bos *et al.*, 1989b), and showed that it closely agreed with the curve obtained by titration.

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Table 2-6, modified from Foster with current analytical data, lists the numbers of potentially ionizable groups and their average intrinsic p*K* values used in reconstructing the titration curves of HSA and BSA from theoretical considerations. The total numbers for each amino acid type are in excellent agreement with to the values from the definitive amino acid composition (Table 2-1), values that were not available to Tanford or to Foster. The agreement is a testimonial to the careful laboratory work by Tanford; it also means that essentially all of the potentially ionizable groups of albumin are accessible to protons of the surrounding solution within the pH range covered by the titration curve. Extensive unfolding of the albumin structure occurs at pH extremes (<3, >10), of course, so the molecule can no longer be considered to be "native." Tanford found the titration curves to be fully reversible— ±0.02 pH unit even after 30 min at pH 12 or 24 h at pH 2 without hysteresis, affirming the resiliency of the albumin molecule.

The most unusual feature of the data of Table 2-6 is the low intrinsic pK values for the β- and γ-carboxyls (aspartic and glutamic acids), half of which average 0.5 pH units less than those typical of other proteins. This phenomenon has been related by Foster to the abrupt expansion of the albumin molecule at about pH 3.8, when it undergoes the **N** → **F** isomerization (Section II,C,1). About half of the carboxyls are considered to ionize with an intrinsic p*K* of 4.3, and the other half below pH 3.7. Thus, there are carboxyls that remain protonated or linked in salt bridges to lysine or arginine residues in the **N** isomer, above pH 4, but become accessible to ionization in the **F** form. The electrophoretic behavior of the peptic fragments 1–307 and 308–583 (Fig. 2-4) also suggests that about three carboxyl groups are hindered from deprotonization in the intact BSA molecule but are ionized when the molecule is cleaved at the 307–308 bond.

The average intrinsic p*K* for the ε-amino groups (lysines) is also lower than generally found in other proteins, and that for phenols (tyrosines) is lower than most of them (Table 2-6). Ionization of the single thiol (CySH-34) is unusually acidic and is discussed in Section II,B,5.

The structural significance of the various altered pK values has been considered by Foster (1960). Although hydrogen bonding between carboxylates and phenols seemed a likely explanation, the entropy and enthalpy changes during titration do not support this conjecture. The explanation must be sought in precise tertiary structure information.

The calculated *distribution* of charges also affects the properties of the albumin molecule. As noted in Section I,C, the calculated net negative charge at pH 7.4 is not uniform among the domains, but is greatest for the amino-terminal domain (domain I) and least for domain III. In the proposed heart-shaped configuration, the top:bottom (or base:apex) distribution is nearly uniform, −6 in the upper half and −9 in the lower half (Fig. 2-9). The electric asymmetry between domains is still evident, however, causing a net charge of −14 for the left half

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TABLE 2-6
Correlation of Ionizable Groups with Titration[a]

Ionizable group	pK[b]	HSA		BSA	
		Titration	Composition[c]	Titration	Composition[c]
β,γ-COOH	4.0	102	98	101	99
α-COOH	3.1	1	1	1	1
Imidazole	4.9–7.5	15	16	16	17
α-NH_2	7.8[d]	1	1	1	1
ε-NH_2	9.2	58	59	56	59
Thiol	5[d]	0?	0.5	0?	0.5
Phenolic	9.6	17	18	19	20
Guanidino	11	22	24	23	23

[a]From titration data of Tanford (1950) and Foster (1960), calculated to 66,500 Da.
[b]From Figge *et al.* (1992).
[c]From Sadler and Tucker (1992).
[d]From Lewis *et al.* (1980).
[e]Composition data from Table 2-1.

and −1 for the right. (The ionization of some groups may be suppressed by nearby residues, but this effect should not be large enough to change the charge distribution markedly.)

J.L. Oncley, of the laboratory of E. J. Cohn, has been the major student of dielectric measurements (1943). The electric asymmetry of albumin is measured by the impedance seen when its molecules align in an electric field, generally at the isoionic pH of the protein (pH ≈5) so that the overall net charge is zero. At this pH the histidine imidazole groups should also be considered as protonated; the calculated net charge of the amino and carboxyl halves of HSA is then −4 and +5, a difference of 9. For the corresponding halves of BSA the calculated net charge is −1 and +1, a difference of 2. The total dielectric increment, D_{sp}, per g/L, of fat-free HSA is about 1.02, and its dipole moment in Debye units is about 700 near 0° (Scheider *et al.*, 1976). For BSA the values are significantly smaller, 0.38 and 420, respectively, which is in accord with its smaller calculated charge asymmetry.

Dielectric effects in alternating fields, from 1 kHz to 100 MHz, give a measure of the rapidity with which a protein molecule can realign when the field reverses. The general model for albumin was considered to be a rigid ellipsoid of major axis about 140 Å and minor axis about 40 Å. Experimental relaxation time constants, τ, about the two axes are ≈0.2 and 0.1 μsec, respectively, at 25°

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with rotary diffusion constants of 4×10^6 and 1×10^6 s^{-1}, respectively, at 0° (Wright and Thompson, 1975; Essex *et al.*, 1977).

Interpretation of dielectric data with the molecule considered to be triangular in shape, and with considerable flexibility, does not appear to have been attempted.

3. Solubility

The solubility of albumins is related to their high total electric charge, with corresponding strong hydrophilicity and attractiveness for water molecules. Near neutrality, albumins are extremely soluble in water or dilute salt solutions; 35% (w/v) solutions are marketed, and 50% solutions can be prepared.

Albumins are "salted out" of solution by addition of more salt. Divalent salts are particularly effective: note the use of ammonium sulfate (King, 1972) or sodium sulfate in classic fractionation methods where albumin is precipitated at about 80% saturation (about 3.5 *M*) ammonium sulfate after removal of globulins at 50% saturation (2.05 *M*). At the isoelectric point, about pH 5, albumin solubility decreases markedly, more than that of most proteins; the repellent effect of like net charge is absent although the total charge remains high.

Albumin is unusual among animal proteins in its solubility in polar organic solvents. Near pH 7 it will remain soluble in pure methanol at room temperature (Pillemer and Hutchinson, 1945) or in 43% ethanol at −5°, conditions that precipitate all other major plasma proteins. Below pH 3 it will dissolve in 99.5% acetic acid (Steinrauf and Dandliker, 1958) or 88% formic acid, as well as in 80–100% methanol, ethanol, or acetone. Less polar solvents such as chloroform or higher alcohols are not effective solvents.

Dilute salt, 0.1 *M*, increases solubility of albumin in ethanolic solutions. Solubility usually rises with increasing temperature in alcohol–water systems (Hughes, 1954); in strong salts, 2 *M*, it may decrease with temperature.

The precipitating action of salts has been proposed to be a competition for the solvent molecules as the salts themselves become hydrated, leaving little solvent available to keep the protein molecules separated from each other. Hydration of salts has been related to their surface tension effects (Arakawa and Timasheff, 1984). Albumins are also precipitated by other water-sequestering substances such as polyethylene glycol or Rivanol (6,9-diamino-2-ethoxyacridine) (Ingham, 1978). Even the action of cold ethanol, formerly attributed to its lowering of the dielectric constant of the solvent, has recently been reinterpreted as one of dehydration, a competition for water molecules (van Oss, 1989).

The theory of protein solubility is treated in the classic monograph of Cohn and Edsall (1943), and Edsall (1947) has published a masterful review of the solubility aspects of plasma protein fractionation. Practical aspects of solubility are considered in Chapter 7, Section I,A,1.

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4. Groups Susceptible to Modification

Chemical groups that are readily susceptible to modification under mild conditions have generally been assumed to be on or near the molecular surface of a protein. An exception to this concept would occur with reagents that first induce a local conformational change, such as those that bind in a specific ligand site, and then react with a nearby constituent. Acetylsalicylic acid, or aspirin, is an example of the ligand type; it binds to the salcylate site, and then transfers its acetyl group to the nearby lysine, shown to be Lys-199 of HSA (Chapter 3, Section I).

This residue 199 has also been identified as one of several HSA lysines shown to be glycated nonenzymatically by glucose (Iberg and Flückiger, 1986) and acyl glucuronides (Ding *et al.*, 1993) *in vitro*. It is specifically modified by sulfonyl fluoride ester compounds with antithrombin activity (Lawson *et al.*, 1982). It is one of two reactive lysines modified by the reagent, 2,6-dinitro-4-trifluoromethylphenyl sulfonate (Gerig *et al.*, 1978), and these two lysines are probably the same as those shown earlier by Green (1963) to be unusually reactive with fluorodinitrobenzene (FDNB). By X-ray diffraction Lys-199 of HSA is found in the hydrophobic binding pocket subdomain IIA, in helix h1 of domain II and near His-242; the influence of His-242 may be responsible for its low pK of 7.9 (Carter and Ho, 1994).

Other reactive ϵ-amino groups of HSA are those of lysines 281, 439, and 525 (aldohexose and glucuronides), lysines 136, 162, and 212 (dansyl chloride), and Lys-195 [acylglucuronide, bromoacetyltryptophan (McMenamy, 1977), or dansyl chloride (Jacobsen and Jacobsen, 1979)]. In BSA Brown and Shockley (1982) found Lys-221, at the tip of loop 4, to be especially reactive with *N*-dansylaziridine and Lys-350 to react with trinitrobenzene sulfonate.

Of all of these reactive lysines the most prominent are Lys-199 (aspirin) and Lys-525 (glucose) of HSA. In interpreting that lysines are truly surface located one must be cautious, considering that Yamada *et al.* (1986) showed with lysozyme that the most readily dinitrophenylated lysines do not correspond to lysines having exposed amino groups in their X-ray crystal structure.

The other group that is highly accessible is Tyr-411 of HSA (Tyr-410 of BSA) and most other albumins. Fred Sanger showed in 1963 that this tryosine is the primary binding site for diisopropyl fluorophosphate; more recently Hagag *et al.* (1983) identified it as the major site for *p*-nitroanthranilate formation, and Peters *et al.* (1988) found it to be the major site of low-level iodination of HSA. The crystal structure places Tyr-411 solidly in the binding pocket of subdomain IIIA, in its h2 helix (Fig. 2-9). Its hydroxyl is said to be 2.7 Å from the Arg-410 guanidinyl nitrogens, the proximity perhaps explaining its ready susceptibility to nucleophilic substitution (Carter and Ho, 1994). Gary Means and co-workers have studied the interesting esterase ability of this tyrosine toward *p*-nitrophenyl acetate (see Chapter 3, Section I.D.6, for further discussion).

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Esterification of carboxyl groups was one of the first modifications tested with albumin (Fraenkel-Conrat and Olcott, 1945). It affected antigenicity more than did modification of amino groups. Recently the "cationization" or addition of positive charges has been of interest in studying passage of proteins through renal membranes or control of the immune process; albumin can be cationized by converting carboxyl groups to amino groups with ethylene diamine (Bass *et al.* (1990). Some general references to the physical chemical and immunological effects of modifying amino and carboxyl groups are those of Coddington and Perkins (1961), Sri Ram *et al.* (1962), Jacobsen *et al.* (1972), Habeeb (1979), and Tayyab and Qasim (1987).

Diazotization, iodination, and nitration affect primarily tyrosyl residues. These are popular sites for conjugation of fluorescent markers and antigenic components. Perlman and Edelhoch (1967) reported that iodination of all tyrosines to diiodotyrosine did not affect secondary structure significantly.

The single tryptophan residue of HSA has been a frequent target. 2-Hydroxy-5-nitrobenzyl bromide is quite selective for the indole group (Fehske *et al.*, 1978), as is photooxidation (Reddi *et al.*, 1987). The alteration modifies the protein configuration only slightly. *N*-Bromosuccinimide is believed to oxidize the 2–3 double bond of the indole ring to form an imino lactone with the carbonyl group; the imino bond splits and cleaves the peptide chain (Peters, 1959b). Cyanogen bromide oxidizes methonyl residues to form homoserine, which likewise results in peptide bond cleavage on lactone formation.

5. *Properties of Thiol Group*

All avian and mammalian albumins for which the structure is known have a single thiol resulting from an unpaired cysteine at position 34 (Chapter 4, Fig. 4-4). The importance of this group calls for special consideration apart from other specific residues noted above. Properties of the 17 S–S-bonded cystines are examined in Section II,C,3.

The thiol of CySH-34 makes up most of the mercaptan of plasma (free cysteine is undetectable, and other plasma proteins contain little or none). As normally isolated from plasma, about one-third of the albumin molecules carry half-cystine or half-glutathione as a mixed disulfide on this cysteine, the ratio being about four to one in favor of half-cystine (Andersson, 1966). These covalently bound ligands are apparently picked up in the circulation, because they are not present on albumin during its secretion from the liver cell. About 4 μM of homocysteine is also found (Fiskerstrand *et al.*, 1993), corresponding to 2% of the bound cysteine.

A preparation of albumin containing no mixed disulfide, in which all of Cys-34 is in the SH or mercaptan form, is termed *mercaptalbumin*, often abbreviated HMA or BMA for the human or bovine species. HMA was first isolated by Hughes of the laboratory of E. J. Cohn, after crystallizing HSA dimers formed by

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linking the single thiols with mercury(II) ion (Hughes, 1954). On removing the mercury with a low molecular weight thiol compound mercaptalbumin was obtained. Shortly thereafter Kay and Edsall (1956) similarly prepared BMA with Hg(II) and reported the kinetics of its formation.

Because the mixed disulfide forms of albumin carry a slightly altered ionic charge, mercaptalbumins can also be separated by ion exchange techniques using a diethylaminoethyl (DEAE) or sulfoethyl (SE) medium (see Chapter 7, Section II,A).

The mixed disulfide formation reaction is reversible, and mercaptalbumins can be prepared by removal of the half-cystine and half-glutathione if the pH is carefully controlled. In the presence of 5 mol/mol (M/M) dithiothreitol (Sogami *et al.*, 1984), or even as much as 200 M/M thioglycolic acid (Katchalski *et al.*, 1957), at room temperature the S–S-bound substances are released and can be removed along with the reducing agent by dialysis or gel permeation methods, yielding mercaptalbumins with 1.0 SH/albumin molecule. Hartley *et al.* (1962) elegantly removed the released mixed disulfide by pumping a solution of 0.01 *M* thiol reagent over HSA that was bound to a DEAE-cellulose column at pH 7. The pH should be carefully held between 5 and 7, however, or disulfide bonds will be reduced and broken. Some salt (≈0.05 *M*) should also be present.

Conversely, mercaptalbumin can be converted into a mixed disulfide form, for instance, half-Cys albumin, in the presence of an excess of a disulfide compound (Chapter 7, Section IV,C). This treatment is often desirable to provide removable protection of the thiol. The exchange reaction with cystine releases a cysteine molecule, which is quickly reoxidized to cystine by dissolved molecular oxygen.

$$\text{Alb–SH} + \text{Cy–SS–Cy} \rightarrow \text{Alb–SS–Cy} + \text{CySH}, \tag{2}$$

$$2\text{CySH} + \tfrac{1}{2}\text{O}_2 \rightarrow \text{Cy–SS–Cy} + \text{H}_2\text{O}. \tag{3}$$

Because free cysteine is easily oxidized to cystine in solution at the pH of blood, the SH of Cys-34 is obviously protected from this oxidation by its situation in the albumin molecule. Considerable investigative effort has been directed toward understanding the properties and molecular environment of this residue.

The sulfhydryl content of albumins was initially measured by amperometric titration with silver ions at pH 7.4. With BSA, 0.67 mol of SH/albumin was found (Benesch *et al.*, 1955). In 8 *M* urea or at 37° the value increased to 1.0 M/M, apparently through removal of the mixed disulfides.

Use of mercuric compounds succeeded the amperometric methods. The reaction was usually detected by a spectral change in an aromatic group bound to the mercury atom; *p*-chloromercuribenzoate (Boyer, 1954), *p*-(2-pyridylazo)dimethylaniline (Klotz and Carver, 1961), and 2-chloromercuri-4-dinitrophenol (Janssen, 1985) are examples.

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The current favorite is the Ellman reagent 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) (Ellman, 1958). At pH 8 it effects a disulfide exchange with free thiols, releasing the SH form of DTNB, which absorbs strongly at 412 nm. For its application see Chapter 7, Section IV,C.

The albumin thiol is also readily accessible at pH 7–8 to alkylating agents such as *N*-ethylmaleimide (Alexander and Hamilton, 1968), iodoacetic acid, iodoacetamide (Brush *et al.*, 1963), acrylonitrile (Weil and Seibles, 1961), or vinylpyridine (Hermodson *et al.*, 1973). The thiol is slowly oxidized by dissolved oxygen on storage (Felding and Fex, 1984), and disappears even in the absence of oxygen at pH <5 and >8 (Simpson and Saroff, 1958); formation of an internal thioester or thiazoline ring is likely. It is oxidized by active oxygen forms such as hydroxyl (·OH), superoxide (O_2^-), and hydrodioxyl (HO_2·) radicals (Davies *et al.*, 1987; Finch *et al.*, 1993), particularly in dilute solution (1 mg/mL) (DiSimplicio *et al.*, 1993). Blocking the SH with a small, uncharged agent such as iodoacetamide causes little or no effect on the secondary structure of albumin (Batra *et al.*, 1989).

The proton of a free thiol normally ionizes above pH ≈9. The albumin thiol, on the other hand, appears to be considerably more acidic, with a p*K* between 5 and 8 (Pedersen and Jacobsen, 1980). Lewis *et al.* (1980), by potentiometric difference titration, found that, although the thiol of BSA in 8 *M* urea shows a p*K* of 8.9, normally its apparent p*K* is less than 5! In either case the Cys-34 sulfur would be in the S^- form at physiological pH.

Studies of the reaction rates with a series of aromatic and heterocyclic disulfides showed that heterocyclic compounds are the most reactive (Mahieu *et al.*, 1993), and that five-membered rings react more rapidly than six-membered rings (Gosselet *et al.*, 1990). From this it was predicted that the thiol of BSA lies in a sterically restricted environment that has a hydrophobic character. Wilson *et al.* (1980) drew the same conclusion using linear disulfide compounds; because a β-amino group on the disulfide compound increased the reaction rate, ion pairing with a carboxylate near the thiol was suggested. Ohkubo (1969) postulated from absorbance and ORD observations on HSA, HMA, and HSA dimer that the thiol sits at a border between a polar helical segment and a hydrophobic area; two tyrosyl groups, one exposed and the other half-buried, may lie nearby.

Electron spin resonance (ESR) measurements using nitroxide compounds of varying length coupled via alkylating agents as "molecular dipsticks" (Cornell *et al.*, 1981; Graceffa, 1983) indicated restriction of movement of the spin label. The thiol was interpreted to reside in a crevice, this time 9.5 Å deep.

The HSA tertiary model provided by X-ray diffraction confirms these predictions (Fig. 2-13 colorplate). Cys-34 is found indeed to be situated in a partially protected site, in the seven-residue turn between helices h2 and h3 of subdomain IA (Fig. 2-11). Tyr-30 lies ≈10.3 Å away, buried in helix h2, and Tyr-84, partially exposed in the short stretch between helices h4 and h5, is only

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≈11.2 Å distant according to Carter (1994). His-39 and Glu-82 are nearby and may influence the pK of the sulfhydryl group. The access opening is about 10 Å.

Details of the site in BSA may differ. The reaction of HSA with [^{14}C]cystine at pH 8 was reported to have a fast component followed by a slow component, whereas the reaction of BSA showed only the fast component (Edwards *et al.*, 1969). The binding of LCFAs appears to affect the access to this site (Chapter 3, Section I,A,3). In the X-ray model, the crevice holding the thiol opens significantly when three or more LCFAs are bound; the Cys-34 to His-39 distance and the exposure to oxygen both increase (Carter and Ho, 1994).

The Cys-34 thiol is not near any internal disulfide links but can be accessible to link with the thiol of another albumin molecule, a reaction probably involving distortion in the form of flattening of the 10-Å pocket. In addition to the mercury–S–S-linked dimers, a direct S–S dimer of HSA has been prepared by gentle oxidation at neutral pH (Andersson, 1970). Polymers were reported after the action of hydroxyl radicals. S–S-linked dimers constitute the majority of the 5–10% of polymeric forms found in most albumin preparations that have been lyophilized during production.

To summarize the properties of the thiol of Cys-34, it is accessible to a mercury atom and to groups the size of a benzene ring, yet is relatively protected from oxidation by molecular oxygen. It sits in a hydrophobic crevice of depth 9.5–10 Å, with a carboxylate group nearby. LCFAs increase the access of oxygen. The thiol itself is normally in the ionized, S form. Tyrosines 30 and 84 lie nearby, one buried and one partly exposed.

C. Changes in Configuration

The albumin molecule undergoes several well-recognized changes in conformation, usually under nonphysiological conditions. These include isomerizations with moderate change of pH, more extensive alterations at extremes of pH or with cleavage of disulfide bonds, and refolding to native configuration after total reduction of these S–S bonds. The changes discussed in this section do not include random molecular motions, which were considered in Section II,A,6, or adjustments at binding sites and allosteric effects on ligand binding, considered in Chapter 3.

1. Isomerizations with Varying pH

Four isomers of the normal, or **N** form, have been recognized—**F**, or fast, at pH 4; **E**, or extended, below pH 3; **B**, or basic, near pH 8; and **A**, or aged, near pH 10 (Fig. 2-14). The accompanying structural changes have been predicted

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through physical chemical evidence and have been only tentatively identified in relation to the now-known tertiary configuration of albumin. All are reversible. The isomerizations are probably of interest more for what they can tell us of the dynamics of the albumin structure than for physiological significance. Most of these isomerizations have been studied with BSA, but they apparently occur in a similar fashion with HSA.

a. F Form. Careful titrations of BSA by Tanford (Section II,B,2) led to the demonstration by Foster that the abrupt discontinuity in titration at pH 4–4.5 coincided with the appearance of a faster migrating, or **F**, form as seen on gel electrophoresis at pH 3–4 (Aoki and Foster, 1957). Increasing amounts of the **F** form appeared as the pH was carefully lowered; he correlated this with the ionization of about 40 COOH side chains with a pK of 3.7, lower than the usual pK of 4.1 (Table 2-6 and Section II,B,2).

Between 1957 and 1962, Karl Schmid, of the laboratory of E.J. Cohn, published a series of papers studying the inhomogeneity of HSA at pH 4 seen with moving-boundary (Tiselius) electrophoresis (Schmid and Polis, 1960), and pointed out that others such as Luetscher had noted this phenomenon as early as 1939. Schmid in particular tested the effects of different anion and cation species on the electrophoretic behavior. The electrophoretic inhomogeneity near pH 4 was well known, but its meaning in terms of isomerization was chiefly elicited by Foster and colleagues.

Foster found that the **F** form of BSA is practically insoluble (<<1 mg/mL) in 3.0 M KCl, whereas at higher pH albumin (i.e., the **N** form) is highly soluble at this salt concentration. Hydrodynamic properties such as sedimentation rate, diffusion constant, and intrinsic viscosity show that the F-isomeric molecule has become longer and increasingly asymmetrical (Fig. 2-14). Ultrasonic absorption and velocity spectra (Choi *et al.*, 1990) more recently have exhibited a relaxation frequency near 200 kHz, attributed to the expansion of the molecule, and another near 2 MHz attributed to the proton transfer reaction of the carboxyl groups.

Harrington *et al.* had shown earlier (1956) that the BSA molecule becomes less compact and has a slower rotational relaxation time. In addition to the sudden availability of 40 carboxyl groups to titration, three to five tyrosyl side chains become unmasked and show a blue shift in their absorption spectrum, as judged by accessibility to bulky solute molecules such as sucrose (Herskovits and Laskowski, 1962; Steinhardt *et al.*, 1971). About 150 more hydrogen atoms become exchangeable with D_2O (Bryan and Nielsen, 1969) or ^3H_2O (Dzhafarov, 1991), and the surface area accessible to solvent increases from 39,000 to 70,400 Å^2. A single disulfide bond becomes reducible by 0.1 M thioglycolate (Katchalski *et al.*, 1957). There is a loss of about 12 binding sites for SDS (Foster, 1960), but a 40–50% increase in fluorescence yield with binding of ANS, which was

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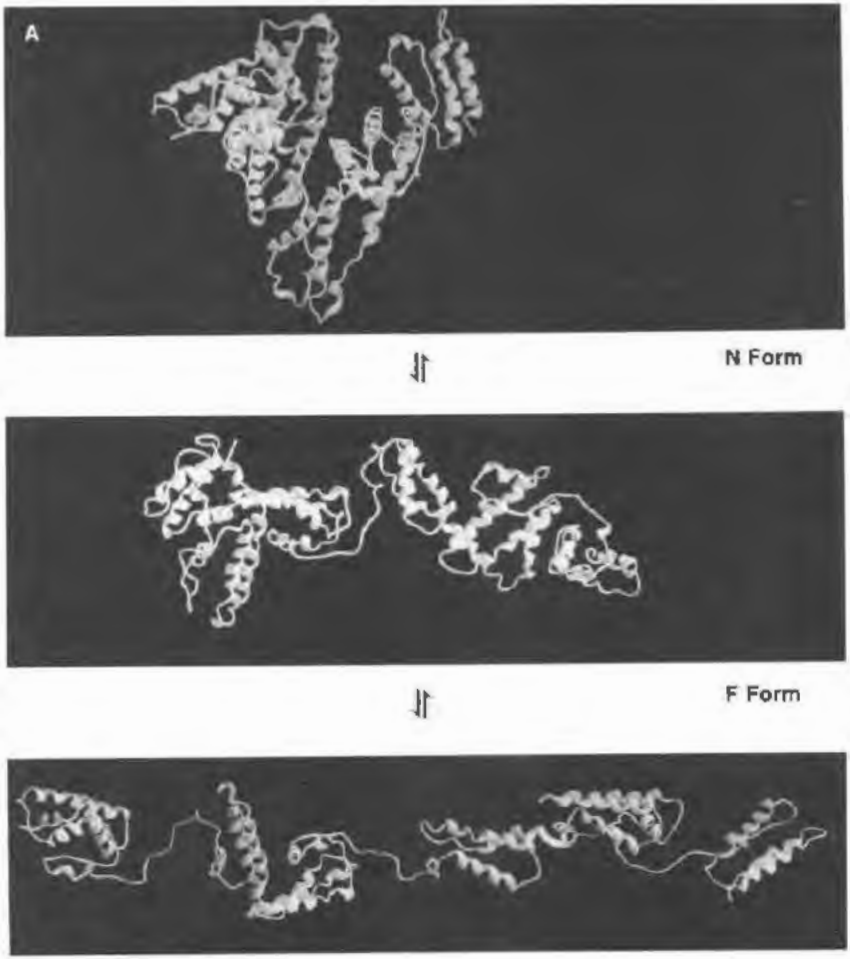


Fig. 2-14. (A) Proposed configurations of F and E isomeric forms of HSA. Reproduced from Carter and Ho (1994) by permission of Academic Press. (B) Interrelation of five recognized forms. Reproduced from Peters (1985) with permission of Academic Press.

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attributed to exposure of additional binding site(s) for this fluorogenic anion (Miller *et al.*, 1991). Helical content decreases, although not sharply, from about 55 to 45% as judged by CD (Era *et al.*, 1983).

"Near all" of the 17 ^{1}H histidine resonances show abrupt discontinuities in their NMR titration curves near pH 4.3 (Sadler and Tucker, 1993). The reaction of the thiol of Cys-34 to form mixed disulfides reaches maximal velocity (Pedersen and Jacobsen, 1980). If this thiol of CySH-34 is labeled with an ESR probe with length enough to extend to the top of the 10-Å crevice (Section II,B,5), an effect on the spin label is seen on passing through pH 4 (Cornell and Kaplan, 1978a). Suzukida *et al.* (1983) reported that the apparent CySH-34 to Trp-214 distance in HSA shortens from 34–35 to 30 Å at pH 3.6.

Foster's former student, Sogami, and others have found that the **F** isomerization proceeds in two steps. The change in helicity observed at 233 nm, and effects on fluorescence of both the HSA tryptophan (Sogami *et al.*, 1982) and a single bound ANS (Era *et al.*, 1985), led to the proposal that the conformational change occurs first to an intermediate **F1** form, which then expands to the **F** form spontaneously, $\mathbf{N} \rightarrow \mathbf{F1} \rightarrow \mathbf{F}$. The whole isomerization measured by stopped-flow analysis requires only about 100 ms (Taylor *et al.*, 1978). It is fully reversible, even from solutions of 8 *M* urea (Kauzmann and Simpson, 1953), within about 1 s (Rudolph *et al.*, 1975).

An **F**-like transformation seen with large fragments of albumin has shed some light on the intramolecular changes. They appear to be most dramatic in the C-terminal region, domain III. The isolated BSA fragment 377–583, domain III (Table 2-2), unfolds and loses helical structure at pH 4 (Geisow and Stuchbury, 1977; Khan and Salahuddin, 1984), whereas fragment 1–387, domains I + II, does not lose helix until pH 3.5 (Geisow and Beaven, 1977; Khan, 1986). Domain III was proposed to have a looser structure than the rest of the molecule, and to expand through separation of its subdomains in the **F** transition.

Foster serendipitously encountered a modified form of BSA in which a single peptide bond was cleaved by an enzyme contaminating some preparations of BSA Fraction V (Wilson and Foster, 1971), and which was active on the **F** form at pH 3.7. The affected bond was found to be in loop 7 of domain III, probably at Gln^{393}-Phe^{394} (Zurawaki *et al.*, 1975), further evidence of the looser structure of this region. This modified BSA entered the **F** configuration more readily, at a pH above 4, and formed a transparent gel at pH 4 at concentrations >70 g/L (Era *et al.*, 1989).

Carter and Ho (1994) propose that the **F** transition is the separation of the two halves of the molecule, domains I + IIA and domains IIB and III, from each other under mildly acidic conditions as demonstrated by the separation of the fragments containing these halves P-B and P-A (Figs. 2-4 and 2-14). Predictably, the separation exposes the peptide cleavage site, Asp^{307}-Phe^{308}, to solvent, whereas Carter (1994) found Phe-308 to be buried in the native structure. The separation accounts for the observed lengthening of the molecule, and the

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grouping of subdomain IIA with domain III provides a total of 42 carboxyl groups to provide the measured ionization of 40 carboxyls.

Mir and Qasim (1986) found an intermolecular effect with BSA oligomers. The midpoints for the **N** → **F** transition in monomer, dimer, and tetramer were pH 3.75, 3.60, and 3.40, respectively. Hence interactions with nearby companion albumin molecules, or the distorting effects of the covalent intermolecular bonds, slow the onset of the isomerization.

*b. **E** Form.* As the pH of a BSA solution is lowered below 3.5, a further unfolding occurs until about pH 2.5, when the molecule appears to be as fully expanded as its disulfide bonding structure allows (Fig. 2-14). Foster termed this the **E**, or expanded, form. At pH 1.7 the intrinsic viscosity rises markedly, indicating a change in axial ratio from 4 to 9, but sedimentation velocity and light scattering indicate no change in molecular weight (Harrington *et al.*, 1956). About three disulfide bonds are now reducible by 2-mercaptoethylamine (Alexander and Hamilton, 1968). Electron microscopy pictures the molecule as a chain of globules, about 21 × 250 Å in size (Slayter, 1965). Following the instant heat evolution caused by the protonation of carboxyl groups there is a slow heat uptake, $t_{1/2}$ = 2 min, of about 2500 cal/mol (Bro and Sturtevant, 1958).

Calculated helicity falls, but only from 45 to 35% between the **F** and **E** forms. Era *et al.* (1983) show higher helicities, from 73% in the **N** form to about 65% in the **F** form to 52% in the **E** form, but the relative declines are about the same. The loss is presumably the intradomain h10 helices (Table 2-4, Fig. 2-9). Whether the residual CD activity is actually due to helical segments preserved by restrictions of the local disulfide bonding or is the result of optical activity of the S–S bonds themselves is not certain.

The average fluorescence of the two BSA tryptophans likewise shows a progressive fall with pH (Brewer *et al.*, 1987). Their fluorescence lifetimes fall from 57 nsec at pH 7 to 44 nsec at pH 4 to 33 nsec at pH 2. The long rotatory correlation times, related to the anisotropy of the tryptophan residues, decrease to about 10 nsec in the fully expanded form. Difference spectra at 287 nm indicated increased exposure of both tryptophans and tyrosines.

The acid expansion is suppressed by increasing ionic strength, an indication that salt forces predominate. When all carboxyl groups have become protonated, the positive charges of lysine, arginine, and histidine residues would cause mutual repulsion between the domains and subdomains of the molecule. Unlike the net charge at pH 7.4, this positive charge is distributed uniformly along the structure; BSA domains I, II, and III (Fig. 2-2) have calculated total charges of +34, +36, and +32, respectively, with charge densities of +1.9, +1.8, and +1.6 per 10 amino acid residues, respectively. Among the nine individual loops, the charge densities per 10 residues average 1.71 ± 0.31. Like the **F** isomerization, the acid expansion is fully reversible.

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c. *B Form.* Leonard *et al.* (1963) attributed the drop in specific rotation, α_{313}, between pH 8 and 9 to a conformational change, which they termed the "neutral transition," from the neutral, or **N**, form to a **B**, or basic, form. The isomerization has also been called the "neutral to base transition" or simply the "basic transition." Like the **F** form, it has its basis in the unusual p*K* of ionizable groups, in this case imidizaoles rather than carboxylates. It is subtler and more gradual in onset, and has been proposed to have physiological significance.

Since 1963 the properties of the **B** isomer have interested many albumin chemists, but chiefly K. Aoki and M. Sogami (two former students of Foster's) and their colleagues at Gifu University and I. Wilting, L.H.M. Janssen, and others in the group at Utrecht. The Gifu laboratory has dealt mainly with BSA and the Utrecht laboratory with HSA. The impression they give is that the **B** isomerization is a structural fluctuation, a loosening of the molecule with loss of rigid ity, particularly affecting the amino-terminal region and heightened by effects of certain ligands.

Physical properties of BSA in the pH range 7–10 had been studied for many years—the titration curve (Fig. 2-12d), chloride binding (Halle and Lindman, 1978), optical rotation α_{233} (Markus and Karush, 1957), reducibility of disulfide bonds (Katchalski *et al.*, 1957), and fluorescence of the tryptophan residues (Steiner and Edelhoch, 1961). UV difference spectra at 287 nm show increased accessibility of some tyrosines (Williams and Foster, 1959) in the pH range 7–9. Tryptophan fluorescence and rotatory relaxation time exhibit little change, however, the latter remaining in the range 71–74 nsec (Rea *et al.*, 1990). About 150 more hydrogens exchange readily with deuterium (Benson and Hallaway, 1970; Hvidt and Wallevik, 1972). Concurrently the exposed surface area enlarges from 39,000 Å^2 only to 47,000 Å^2 (Dzhafarov, 1991); Tyr, Ser, Arg, Gly, Ile, Phe, and Pro residues are particularly exposed. Disulfide exchange reactions are evident, but can be prevented by alkylation of CySH-34 with iodoacetamide (Sogami *et al.*, 1969). Molar ellipticity calculations from near-ultraviolet CD (Wilting *et al.*, 1979; Era *et al.*, 1990) estimate only a small loss of helix (10%) and gain in β structure (8%). The latter workers used alkylated BSA to prevent the complication of disulfide interchange (see **A** form, Section II,C,1,d). They also studied the confusing effects of ionic strength; ellipticity loss is greater in 0.2 *M* KCl or without salt than it is in 0.1 or 0.15 *M* KCl.

Early in the study of the **B** isomer Harmsen *et al.* (1971) of the Utrecht group found an effect of calcium on the **N** → **B** transition, 0.002 *M* Ca^{2+} causing the midpoint of the transition as judged by α_{313} to fall from pH 8.36 to 7.68. This brings the onset of the structural change into the range of pH encountered in blood plasma, and is the basis for suggestions that the transition has physiological significance in ligand transport.

More definite changes have been apparent in the binding of ligands. The fluorescent ligand, ANS, showed a drop in rotatory relaxation time from 131 to 114

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nsec between the N and B forms of BSA (Era *et al.*, 1990). The binding of warfarin showed increased fluorescence and increased affinity as measured by CD at 310 nm, suggesting that this ligand is more spatially confined (Kasai Morita *et al.*, 1987). The increased affinity can even be observed in whole serum (van der Giesen and Wilting, 1983).

The effects on warfarin binding, including the influence of added calcium ion, are as evident in HSA fragment 1–387 as in whole albumin, and are absent in fragment 195–585 (Bos *et al.*, 1988c). Other compounds known to be bound in the region of subdomain IIA ("Site I"), such as quinone (Wanwimolruk and Birkett, 1982), bilirubin (Jacobsen and Faerch, 1981; Koren *et al.*, 1982), and aflatoxin B1 (Dirr, 1987), also show increased affinity, whereas several drugs bound to the region of subdomain IIIA ("Site II")—indomethacin, salicylate, and phenytoin—show no effect of the N → B transition (Wanwimolruk and Birkett, 1982). Diazepam, however, which is bound in Site II, shows increased affinity up to pH 9 and a strongly allosteric effect (Dröge *et al.*, 1982); the CD changes of bound nitrazepam predict two distinct steps during the transition (t' Hart *et al.*, 1986).

The interpretation of the effects on ligand binding is that the structural changes in the basic transition occur in domain II, with a contribution from domain I, and that the change in domain III is small. This interpretation is reinforced by Lapresle (1990), who found with monoclonal antibodies specific for different HSA domains that the effects of the N → B transition *in whole serum* are strongest in domains I and II and are slight in domain III.

Effects on particular residues support this concept. ^{1}H NMR chemical shifts showed several histidine C-2 atoms to have unusually high pK values, being protonated above pH 7 in the N form; five of these were seen in domain I and were affected by calcium, causing Bos *et al.* (1989b) to assign them a dominant role in the transition; they excluded His-3, so the five would be histidines at positions 9, 39, 67, 105, 128, or 146. Cysteine-34, when probed by NMR using a ^{19}F tag (Zurawski and Foster, 1974) or spectrally with 2-chloromercuri-4-dinitrophenol (Janssen, 1985), showed involvement between pH 8 and 9 with a calcium effect; the NMR results indicated that this proceeded in two steps. Cornell *et al.* (1978b), using an ESR probe long enough to fill the 10-Å crevice, found that the crevice opens during the transition as evidenced by a decreased relaxation time. The distance between CySH-34 and Trp-214 of HSA shortened slightly, from 34–35 to 31 Å (Suzukida *et al.*, 1983), and from the primary bilirubin site (Arg-222?) and Trp-214 from 24.4 to 23 Å (Honoré and Pedersen, 1989).

Bos *et al.* (1989a) predicted before the X-ray crystallographic results were known that domains I and III are in close proximity and that albumin very probably has a U-shaped structure. This was based on the probability of salt bridges between domains I and III and the short distance determined fluorometrically between Trp-214 and Tyr-411 (Site II). Era *et al.* (1990) cited the lengthening of

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this distance from 18 to 24 Å in the **B** form as an indication of increased separation of the two terminal domains, and Bos *et al.* noted that the conformational change in fragment 1–387 (domains I + II) is not exactly the same as in whole HSA, so that there is some influence on domain III. The composite picture from these two laboratories is that the basic transition involves delayed deprotonation of five histidines in domain I, with breaking of salt bridges from domain I to domain III, causing increased flexibility of the molecule and tighter bonding to ligands in subdomain IIA. The Utrecht group (Bos *et al.*, 1989b) suggested that, although domain I is not a major ligand-binding region, its conformational change might be a factor in releasing ligands incident to transport across membranes (see Chapter 5, Section II,B).

d. A Form. In 1969 Sogami *et al.* (1969) found an unexpected new band on electrophoresis of some defatted BSA that had been left in a salt-free solution in the refrigerator for 3–4 days at pH 9. It migrated more slowly than the **N** form, indicating a higher isoelectric point, and amounted to about half of the albumin in the preparation. It was shown to be the result of a sulfhydryl-catalyzed disulfide interchange reaction, being blocked if the albumin sulfhydryl was alkylated and accelerated by small amounts of free thiol compounds. Its band on electrophoresis and its solubility curve at pH 3 show greater heterogeneity than the **N** form.

Nikkel and Foster (1977) named this the **A**, or aged, form (a somewhat unfortunate choice, because "Albumin A" had already been used to denote the common HSA allotype; see Chapter 4, Section IV). Sogami and co-workers at Gifu University have been most active in subsequent studies (Kuwata *et al.*, 1994). The requirement for alkaline conditions indicates that the ionized form of the sulfhydryl, S−, is the active participant. At pH 8.6, with $\mu < 0.02$, conversion of pure defatted bovine mercaptalbumin from the **N** to the **A** form is complete in 10–15 h at 25°. The **A** configuration does not form below pH 7, but will persist for about 20 h at pH as low as 6.5 if it was formed at higher pH. Wallevik (1976) demonstrated that the "aging" reaction proceeds in two steps, given by Eq. (4),

$$\mathbf{N} \rightarrow \mathbf{A}_1 + m\mathrm{H}^+ \rightarrow \mathbf{A}_2 + n\mathrm{H}^+, \tag{4}$$

with isoelectric points of 5.39 and 5.45, respectively, for the $\mathbf{A}_1$ and $\mathbf{A}_2$ forms of defatted BSA, and observed that the isomer even reverts to the **N** form *in vivo*. The source of the released protons has not been pinpointed, but ultrasonic spectroscopy showed peaks at 2 and 15 MHz that were attributed to loss of protons from phenolic and amino groups, respectively (Choi *et al.*, 1990). The five or more imidazole groups with abnormally high p*K* in the **B** isomerization (Section II,C,1,c) must also contribute protons, despite the report by Stroupe and Foster (1973) of ideal behavior for the histidines of the **A** form.

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There is a concomitant decrease in the fluorescence of the tryptophans (Inouye *et al.*, 1984) and depressed solubility in 3 *M* KCl at pH 4, with little loss of helical structure beyond that seen in the **B** form (Era *et al.*, 1991). The isomerization is markedly slowed when conducted in heavy water, D_2O, an environment that strengthens hydrogen bonding (Kuwata *et al.*, 1985), and also by the presence of salt. Even 0.03 *M* KCl lowers the fraction of the **A** form from 0.60 to 0.24 in solutions kept at pH 8.6 overnight at room temperature; calcium and other divalent cations are even more effective. These findings indicate changes in both ionic forces and hydrogen bonding in the **N** → **A** transition. Modest increases in exposure of chromophores and fluorophores to solvent and in susceptibility to proteolysis indicate a more open conformation than the **N** form at neutral pH (Stroupe and Foster, 1973).

The transition is "almost" reversible (Kuwata *et al.*, 1994); at pH 8.6, 0.02 *M* KCl, and 25° an equilibrium value of 30% **A** form is reached in 5 h. Increasing the KCl concentration to 0.1 *M* or adding 2 m*M* $CaCl_2$ lowers the equilibrium value to about 10%. ClO_4^- ion blocks formation of the **A** species more strongly than does Cl^-, leading to the suggestion that the anion effect is the result of binding to the albumin, because the chlorate is bound more strongly than chloride. Medium-chain fatty acids (MCFAs), C_6 and C_8, impede the formation, and C_{10} and C_{12} fatty acids or acetyltryptophan block it entirely; the stability provided by these organic ligands apparently prevents the structural change.

Foster found that the remaining cysteine residue is still in fragment 1–183, and suggested that the S–S bonds that participate in the interchange reside in domain I. Beeause it would be expected that the **A** isomerization is an extension of the **N** → **B** transition in which structural alterations occur primarily in domains I and II, the aging reaction might be considered as a covalent fixation of the structural expansion of the **B** form throngh local shuffling of S–S bonds.

Thermodynamic studies by Kuwata (1994) showed ΔF of nearly 0 kcal/mol for the transition, which led these authors to conjecture that albumin might be "alloplastic" (a term coined by Klotz) and have more than one stable state, a slight modification to the concept of Anfinsen that protein configuration is entirely dependent on amino acid sequence. A loss of several protons, however, surely is a change in composition sufficient to account for a minor change in conformation. The same authors also found the free energy of activation to be about 24 kcal/mol, close to the value of ≈20 kcal/mol for the *cis–trans*-proline isomerization, which they felt may indicate a contribution of this isomerization to the **N** → **A** transition.

2. Denaturation

There is a massive literature conceming the behavior of albumin under various denaturing conditions owing to its role as a model protein. Some salient fea-

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tures are given here rather than a comprehensive coverage, with apologies to the many brilliant protein physical chemists whose results may not be cited; their work is still part of the basis on which current understanding is built.

How should we define "denaturation"? Formerly equated with irreversible loss of secondary structure and biological activity, denaturation is now regarded as any major change from the native structure "that is noncovalent, cooperative, and reversible, in principle if not in practice" (Tanford, 1968; Dill and Shortle, 1991). It is recognized to contain two subsets of microstates—an "unfolded" subset with high exposure to the solvent, and a "compact denatured" state under less strenuous conditions, which still contains considerable structure. This definition is attractive in its parallel to unfolded states encountered in peptide chain synthesis and biosynthesis and to partially altered configurations that are targets for proteolysis during degradation (Dill and Shortle, 1991). It does not equate with a totally random coil conformation.

Albumin, although a fairly complex, multidomain molecule compared to many proteins, can recover from changes in structure caused by almost any conditions other than heat plus strong alkalinity. Even the complete cleavage of its disulfide bonds is reversible (see Section II,C,3). With albumin we will include as "denaturation" unfolding or major structural change caused by one or more conditions usually considered denaturing—chaotropic solvents, organic solvents, extremes of acidity or alkalinity, heat, or spreading at an interface.

a. Solvents. The commonly used denaturing solvents, urea and guanidinium chloride (GuCl), in high concentration weaken hydrophobic interactions by causing water to act as a better solvent for nonpolar residues, and weaken polar interactions by competing for hydrogen bonds. As "good" solvents, they characteristically result in increased viscosity and increased rms radius of a protein molecule according to the ratio (Dill and Shortle, 1991) given by Eq. (5):

$$\text{Radius} = k(\text{number of amino residues per chain})^{0.67}. \qquad (5)$$

With albumin there are few effects below a urea concentration of 4 *M* or GuCl of 1.8 *M* (Khan *et al.*, 1987); then changes in CD, UV, and fluorescent spectra occur stepwise to about 8 *M* urea, consisting of a rapid initial change followed by molecular expansion (Chmelik *et al.*, 1988). The midpoint of the optical changes is about 6 *M* urea, and of the viscosity increase about 5 *M* (Gutter *et al.*, 1957). Maximum change in reduced viscosity is from about 0.14 to 0.2 at pH ≈5; a doubling of intrinsic viscosity is not seen until 8 *M* urea and pH near 10 (Frensdorff *et al.*, 1953). As the urea concentration is raised the titration curve at 7 *M* urea shows an increased affinity of carboxylates for protons, but no change in the total hydrogen ion uptake (Levy, 1958).

There is a gradual onset of S–S bond cleavage by thiol reagents with increasing concentrations of urea, with full reduction of the 17 bonds seen in 8

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M urea of pH 5 (Kolthoff *et al.*, 1960). Large hydrophobic regions still remain at intermediate degrees of cleavage, which disappear only if all the disulfide bonds are broken.

Monitoring the fluorescence of large fragments of BSA, Khan *et al.* (1987) proposed that changes with urea occur first in the more loosely folded domain III, indicating an unfolding or separation of its subdomains at about 4 *M* urea. Minor changes occur in domain II at this concentration. Note that this is the same molecular region in which the F isomerization occurs at pH 4 (Section II,C,1,a). Domain III is also the region where long-chain fatty acids first bind; albumin containing 1–2 M/M fatty acid is more resistant to changes with 8 *M* urea (Rosseneu-Motreff *et al.*, 1973).

The effects of 8 *M* urea or 4 *M* GuCl on BSA (pH 5, 25°, for up to 5 days!) are completely reversible as judged by viscosity, helicity, and nonavailability of S–S bonds for reduction *if* the albumin concentration is low—2.5 mg/mL or less (Kolthoff *et al.*, 1960). At higher concentrations the oligomers remain, seen as a rise in viscosity. Because aggregation occurs chiefly with mercaptalbumin, it is attributed mainly to S–S bond interchanges (Chmelik *et al.*, 1988). Alexander and Hamilton (1968) found full reversibility after exposure to 5 *M* GuCl between pH 5 and 9 if S–S bond interchange had been prevented by alkylation of CySH-34. Batra *et al.* (1989), however, noted that this alkylation caused CD changes at lower than usual urea concentrations.

At BSA concentrations of 50 mg/mL at pH 7 (Maurer, 1959), a gel formed 16 h after removal of urea by dialysis; the fraction soluble at pH 5 appeared native by viscosity and optical rotation but contained about 10% dimer. Its reaction with anti-BSA antibody was depressed about 15%, indicating minor folding changes.

At low pH albumin dissolves readily in many organic solvents (Section II,B,3). The molecule expands, probably owing to weakening of hydrophobic bonding, but returns to native structure on removal of the solvent. Near neutrality, however, albumin undergoes an aggregation in ethanol that is preventable by blocking the thiol group (Rosenberg *et al.*, 1962). It is the only plasma protein remaining soluble (if not undamaged) in methanol at pH 7 as used in an earlier assay procedure. With increasing temperature and pH, organic solvents cause an irreversible denaturation, concern for which is the basis of the strict use of subzero temperatures during exposure to ethanol in albumin purification from plasma.

Less polar organic solvents that are miscible with water act as denaturants. At low concentrations the solvent molecules associate with hydrophobic residues; BSA binds 2-chloroethanol, for example, so strongly that the increase in hydrodynamic molecular size is readily measurable (Maes, 1976). Such solvents are helix inducing, and at increased concentrations can drive proteins into highly helical states as judged by CD.

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The action of detergents is complex. Large anionic detergents, e.g., SDS, cause unfolding and increased accessibility of S–S bonds to reduction. Six tyrosines of HSA show a red shift in absorbance when dodecyl sulfate is added (Zakrzewski and Goch, 1968) (see Section II,B,1,a for their possible residue numbers). The fluorescence spectrum of BSA shows quenching at 5 equivalents of SDS and a blue shift at 12 equivalents (Halfman and Nishida, 1971a). In high concentrations detergents associate fully with aliphatic residues and allow estimation of molecular size on electrophoresis in gels. Cationic detergents likewise can cause increased S–S exchange; here the mechanism may be an increased availability of the thiol of CySH-34 from repulsion of cationic residues in its 10-Å crevice (Hiramatsu, 1977). A high loading, $\bar{\nu}$ of 50, is required for denaturation as judged by CD (Nozaki *et al.*, 1974).

b. Extremes of pH. A major gain or loss in hydrogen ions results in large net positive or negative charges with accompanying static charge repulsion. Aoki *et al.* (1973) observed from Raman spectral changes that some of the S–S bonds change from the *gauche–gauche–gauche* to the *gauche–gauche–trans* forms on unfolding at low or high pH.

The changes down to pH 2 were discussed in Section II,C,1,b (the **E** isomerization). They are analogous to the effects of urea—expansion with some loss of helix—and are fully reversible if brief. After exposure to pH 1.2–3.5 for 24 h at 0° there was no increased availability of S–S bonds to reduction on subsequent return to pH 6; only one S–S bond was cleaved in 24 h at pH 3 (Katchalski *et al.*, 1957). Precipitation with 5% TCA (pH ≈0.8) did not affect the optical rotation, viscosity, sedimentation rate, or acid titration curve of BSA after redissolving at pH 7 (Rao *et al.*, 1965). Exposure to pH 1.5 at room temperature for 5 weeks, however, caused loss of immunological reactivity (Maurer, 1959) and probably aspartyl bond cleavage as well.

Effects of alkaline conditions are generally minor until pH 9 (see discussion of the **A** transition) but quickly become more marked and less reversible as the pH increases. Between pH 7 and pH 10 three disulfide bonds are reducible in BSA, five in HSA (Katchalski *et al.*, 1957); at pH 11 this increases to six (Alexander and Hamilton, 1968). If 5 *M* GuCl or 8 *M* urea is then added all bonds are reduced. Although the normal ionization of the tyrosyl hydroxyl group is at about pH 10.3, only one-third of the 18 tyrosines of HSA are deprotonated at pH 11.3, compared to the 16 that would be predicted (Steinhardt and Stocker, 1973; Honoré, 1987). Four of these show rapid changes, 300 s^{-1}, and two less rapid, 57 s^{-1}. Another third become ionized at pH 11.8, but full ionization of tyrosine residues requires pH 12.7 (Eisenberg and Edsall, 1963). These studies monitored the ionization by the marked shift from 287 to 243 nm of the absorption maximum of tyrosine on deprotonation.

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The unfolding of the molecule takes time, and is speeded by high concentrations of detergent. It is commonly monitored by the stopped-flow technique. Instantaneous changes on raising pH are interpreted as involving already exposed tyrosines. The irreversible conformational changes caused by exposure to alkali proceed through a series of intermediates (Aoki *et al.*, 1973; Wetzel *et al.*, 1980), believed to signify the serial involvement of domains or subdomains in disulfide interchange initiated by Cys-34. The progression can be prevented, or at least slowed, by alkylating this free thiol. With strong alkalinity, S–S bonds are broken to form S^- groups, which are oxidized directly by dissolved oxygen (Noel and Hunter, 1972; Wallevik, 1973b); at 0.2 *M* NaOH and 0.01 mg/mL, five S–S bonds were severed (Florence, 1980). Irreversibility is evident after exposure of BSA to pH 13.0, 1.5 h, 0°; after return to pH 6 two disulfide bonds were reducible, whereas after pH 10.2 there was no change (Katchalski *et al.*, 1957). After exposure to pH 11.5 for 30 h at room temperature, however, there was residual increase in viscosity, loss of antigenic activity, and an "odor of sulfur" (Maurer, 1959). As this smell tests suggests, the changes in alkali are related to disulfide interchanges. If the albumin concentration is moderately high (>10 mg/mL), aggregation through intermolecular bonding is severe. Most of these changes can be prevented by alkylation of the thiol group.

c. Heat. With rising temperature there is increased intramolecular motion, allowing facile jumping over free-energy hurdles to numerous structural variations. Particularly with increased albumin concentration occur intermolecular aggregation (Gallier *et al.*, 1987) and irreversible structural alterations. The temperature is also a major factor in the severity of changes seen with other denaturing conditions.

Albumin is amazingly tolerant of high temperature under certain conditions. Recall that all commercial human (and even bovine) albumin preparations have been "pasteurized" by heating at 60° for 10 h (Chapter 7, Section I,B,4) to inactivate pathogenic viruses, and appear essentially unchanged by this treatment. Yet, at pH 9, a 1-min exposure at 65° causes irreversible loss of helix and polymerization (Aoki *et al.*, 1973), as does exposure to 8 *M* urea at 44° (Tanford, 1968). A fairly recent modification to the plasma fraction techniques is the use of "heat shock," heating to temperatures above 60° in the presence of low concentrations of organic solvents, which readily removes most globulins (Chapter 7, Section I,B,1).

Wetzel *et al.* (1980) have investigated the effects of heat on HSA in a meticulous fashion. Loss of α helix (61 to 44%) and gain of β form (6 to 16%) occur between 62° and 75° according to both CD, infrared, and laser Raman (Clark *et al.*, 1981b) studies. If the static charge effect at pH 2.8 (E form of BSA) is added to the effect of a temperature of 63°, the helical content falls beyond 44% to 32% (Takeda *et al.*, 1989).

NMR shows a ^1H time constant increase beginning at 52° that is faster at 62° and indicates denaturation at 72° (Gallier *et al.*, 1987). Electron microscopy

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of thin sections of BSA gels (Clark *et al.*, 1981a) shows a "string of beads" of linearly oriented globules; small-angle X-ray scattering similarly indicates a linearly directed aggregate of unfolded molecules (Clark and Tuffnell, 1980). Availability of S–S bonds rises from 5% at 60° to 47% at 100° (Alexander and Hamilton, 1968). Maximal denaturation was reported at 110–120° with cooperative destruction of postdenaturation remnants (Kazitsyna and Sochava, 1990).

The time of exposure is particularly critical at higher temperatures. Only 3% of BSA will precipitate on removal of salt after heating at 80° (pH 7, 0.13 *M* phosphate) for 20 s, but the precipitated fraction rises to 70% at 2 min and 98% at 4 min (Alexander and Hamilton, 1968). The effects of heat up to 45° (Takeda *et al.*, 1989) or to 20% of maximal denaturation (Wetzel *et al.*, 1980) appear to be fully reversible. After 80° there is still 60% reversibility. Concentration effects are important. Even the loss of intramolecular helix has a midpoint 5° lower at 0.5 mg/mL than it does at 0.05 mg/mL. Formation of 26–36S aggregates (molecular mass $>10^6$ Da) at 80° is 100% at 10 mg/mL but only 48% at 0.5 mg/mL. Intermolecular bonding through β structures leads to aggregation that is reversible to 70°, and preventable by the addition of salt in high concentration (Warner and Levy, 1958). Blocking of the thiol with *N*-ethylmaleimide prevents irreversible aggregation to 75°, and iodoacetamide treatment prevents coagulation of whole serum at 100° (Jensen *et al.*, 1950).

Differential scanning calorimetry (Yamasaki *et al.*, 1990) has helped to understand the mechanism of heat denaturation. Fat-free, SH-blocked BSA exhibited two peaks as temperature was raised, indicating a transitional stage. The enthalpy increased with ionic strength in the neutral to mildly alkaline range. With 0.2 *M* NaCl there was no change in fluorescence of HSA tryptophan or bound ANS (Niamaa *et al.*, 1984) up to 50°. In the presence of lithium bromide, 6.18 *M*, the specific rotation was invariant at temperatures as high as 90° (!) (Harrington and Schellman, 1957), indicating to the authors that the salt did not decrease the intramolecular hydrogen-bonded structure appreciably, but rather that it *increased* the strength of the peptide hydrogen bonds. The thermostability inherent in salt solutions was greater with more chaotropic species, chlorate > isothiocyanate > bromide > chloride (Damodaran, 1989). According to Yamasaki *et al.* (1991), heat induces electrostatic repulsive forces, particularly in the narrow stretch of the albumin chain between Arg-185 and Arg-217, in ligand-binding Site I.

The biphasic nature of temperature–denaturation curves can also be the result of migration of stabilizers. Octanoate, long-chain fatty acids, and *N*-acetyl-L-tryptophan protect HSA from denaturation at 60° even at 5% albumin concentration (Boyer *et al.*, 1946; Edsall, 1984). Concentrations of 4 m*M* are optimal for octanoate (the most effective agent) and *N*-acetyl-L-tryptophan (Yu and Finlayson, 1984a). As with other denaturing conditions, the fat-free, unprotected form is the species that is readily susceptible to heat and will aggregate

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and precipitate at 63° (Shrake *et al.*, 1984; Shrake and Ross, 1988); as the fatty acids dissociate and move among albumin molecules, those that are temporarily fat free become denatured (Gumpen *et al.*, 1979; Aoki *et al.*, 1984). The remaining molecules accumulate fatty acids at higher molar ratios and become highly resistant to heat (or urea) (Brandt and Andersson, 1976). Octanoate bestows a 22° additional heat stability, and palmitate, 15°. Because aggregation requires time, the scan rate of scanning calorimeters is an important consideration in obtaining reproducible results.

Denaturation of fat-free albumin by heat is also dependent on its concentration, being a process of aggregation. Denaturation of albumin carrying fatty acids, on the other hand, is unrelated to concentraton (Ross and Shrake, 1988). The fat-free form is also more sensitive to excursions of pH from neutrality (Gumpen *et al.*, 1979), and is the form that is protected to some extent by the presence of salt.

With extreme conditions of heat and time, especially under alkaline conditions, covalent changes to the amino acid residues of proteins become detectable. In lysozyme at pH 8 at 100°, for example, 18% of Asn residues are deamidated to Asp per hour (Adhern and Klibanov, 1985) (Gln is apparently more resistant to deamidation by heat). In albumin these conditions cause loss of disulfide bonds with a half-life of 0.9 h, via beta elimination to form dehydroalanine and thiocysteine (Volkin and Kilibanov, 1992).

Ultraviolet irradiation likewise results in some covalent changes. A germicidal UV lamp in 3 h caused peptide bond cleavage in BSA with attendant loss of immune reactivity (Maurer, 1959). Other radiation-induced changes are considered in Section II,C,3.

d. Surfaces. As interest in plastic optic lenses and coating of *in vivo* prostheses grows, so has the intensity of study of surface effects on albumin and other proteins. The amount of a protein, including albumin, that occupies most surfaces in a monomolecular film is near 0.15 $\mu g/cm^2$ (Mura-Galelli *et al.*, 1991). As far back as 1947, Bateman found a figure of 0.13 $\mu g/cm^2$ during the use of films as an assay procedure for plasma proteins, and Bull (1947) in his review reported a figure of 0.135 $\mu g/cm^2$.

Fluorescent X-ray interference patterns show that BSA molecules in monolayers lie with their short axes perpendicular to the surface (Sasaki et al., 1994). The molecules are flattened, the spreading being considered the result of surface tension forces that cause stretching of components of the normal conformation. At 0.15 $\mu g/cm^2$, the *calculated* area of an albumin molecule on a surface is 7070 $Å^2$. This implies that a triangular albumin molecule with equilateral 80-Å sides and a 29-Å average thickness has become flattened to 127-Å sides and an 11.6-Å average thickness, a flattening of 2.5-fold.

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Kinetics of binding to a surface include an initial rapid phase (Damodaran and Song, 1988) followed by a slower approach to equilibrium. Predenaturation or unfolding of the molecule will accelerate this phase. The standard free energy of transfer, ΔG_0, is reported as 9.2 kcal mol^{-1} for any protein on any surface (Hajra and Chattoraj, 1991). FTIR, CD, and ellipsometry (Wu *et al.*, 1993) show a loss of α helix and gain of random coil on adsorption (Lenk *et al.*, 1989) at equilibrium, with an intermediate state of β structure.

Denaturation enthalpies have been measured by microcalorimetry of BSA on alumina (Filisko et al., 1986). Reflectance fluorimetry of BSA tryptophan indicates a decrease in both quantum yield and fluorescence lifetime (Rainbow *et al.*, 1987). The absorbed BSA is proposed to contain microaggregates and partially unfolded molecules in a loosely held layer, beneath which is a tightly held layer in a still further-unfolded state.

The distribution of the absorbed albumin varies with the hydrophobicity of the surface; on glass the distribution is homogeneous whereas on more hydrophobic materials the albumin tends to group in islandlike structures (Uniyal and Brash, 1982). Kulik *et al.* (1991) propose that adsorption on quartz initiates at numerous centers; a subsequent lateral motion of adsorbed albumin was detected on polymethylmethacrylate if less than 69% of the surface was covered (Tilton et al., 1990).

Spreading into bubbles of a foam is generally detrimental to protein structure. Here the interface is liquid–air rather than liquid–solid. "Foamed" BSA, however, showed little change in CD and no increase in oligomer; there were some differences in tryptophan fluorescence emission on spreading between these two flexible media (Clark et al., 1988).

Looking back at albumin denaturation, we see that mild loss of α helix and perhaps some gain in β structure are common, together with mutual repulsion of subdomains by unusual static charge conditions. There are similarities in the effects of urea and acid, although the mechanisms differ, urea weakening hydrophobic interactions and hydrogen bonding, and acid causing static charge repulsion. Increased surface of the protein is accessible to the surrounding solvent, exposing more and more side chains of amino acids to its effects. More than in most proteins, in albumin these changes are reversible except in the combined presence of alkali and heat. Complete unfolding requires opening of S–S bonds, discussed in the following section.

3. Breaking and Reforming Disulfide Bonds

The 17 disulfide bonds of mammalian albumins are aligned in a serial fashion along the peptide chain, following the single thiol of Cys-34 (Fig. 2-1). Their overlapping conformation at the paired cystines and the native loop configurations

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provide stable structures with little strain on the S–S bonds. Hence it is no surprise that these cystine bridges do not appear to be labile under physiological conditions, and that albumin has the capability to regain its structure following their rupture.

a. Breaking Disulfide Bonds. Even with exposure to 0.2 *M* thioglycolic acid there is no reduction of disulfide bonds in the pH range 5–7 with salt present and denaturing agents absent; only mixed disulfide compounds attached to Cys-34 are affected (Katchalski *et al.*, 1957). Carter and Ho (1994) relate this stability to the buried location of all 17 disulfide bridges. As the pH moves above 7 or below 5 there is a first a gradual onset of S–S bond reduction; at pH 7.38, for instance, the measurable thiol is 2 M/M albumin (the even number implies the presence of at least one mixed disulfide formed with the thioglycolate reagent, or reduction of one or more S–S bonds in a fraction of the albumin molecules). The number of bonds reduced climbs rapidly in the pH ranges 3–4 and 8–10 (Habeeb, 1979).

Full reduction requires the presence of detergent (Hunter and McDuffie, 1959), 8 *M* urea (Kolthoff *et al.*, 1960), or 4–6 *M* GuCl (Katchalski *et al.*, 1957) at pH 8–9 along with 0.05–0.1 *M* thiol reagent. It is usually conducted overnight at room temperature.

Reduced albumin preparations may be stored below pH 2, even in 0.1 *M* HCl, or as a precipitate with TCA. In order to study them at higher pH, however, the thiols must be blocked, customarily by carboxymethylation with iodoacetic acid or iodoacetamide.

Sodium borohydride has been used as an alternative reductant to thiols, but it is prone to cleave peptide bonds as well (Andersson, 1969). Another useful reagent is sulfite, which can achieve complete disulfide bond cleavage with the generation of SSO_3^- groups in place of thiols. Kella *et al.* (1988) have reduced BSA with 0.1 *M* sodium sulfite, pH 7, with 1.5 m*M* Cu(II) and adequate oxygen for time periods between 2 and 300 min, and observed stages with *average* numbers of 4, 7, 10, 14, and 17 S–S bonds cleaved.

In these preparations, specific viscosity increased from 0.05 to a maximum of 0.4 at 14 bonds cleaved, and then declined to 0.15 at full reduction. Likewise there was a gradual loss in α helix and an increase in β structure, but 15% α- helical structure remained at full reduction as measured by CD. Solubility in the pH range 3–5 decreased gradually and reached a minimum in the preparations with 10 or more bonds cleaved. Fluorescence with bound ANS, intrinsic fluorescence, and UV difference spectra decreased in a similar manner. The changes were interpreted to indicate an increased flexibility of the molecule.

Whether the increasing cleavage of S–S bonds in albumin with increasing time, urea concentration, or pH represents all-or-none reduction of some of the

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molecules or, alternatively, stepwise reduction by domains or regions of all of them has not been resolved. The stages observed (but not isolated) by Kella *et al.* (1988) may represent stable intermediates with partial reduction. Habeeb (1979), however, found albumins after varying degrees of reduction to show only two components on Sephadex G-200 or electrophoresis, corresponding to native and extended forms. This finding favored the all-or-none mechanism of albumin reduction. Note below, however, the "molten globule" state observed with reoxidation.

Radiolytic cleavage—exposure to γ radiation in the presence of formate—caused reduction of 75% of the S–S bonds of BSA when ≈30 rads of ^{60}Co or ^{137}Cs radiation were applied at pH 4 with 100 m*M* formate (Koch and Raleigh, 1991). Hydrated electrons are proposed to be involved in a chain reaction. High-voltage electrons (Alexander and Hamilton, 1968) and X or γ-rays (Yalow and Berson, 1957) have also caused SH groups to appear. Side effects such as peptide bond breakage were not reported.

Oxidation of disulfides is usually carried out by treatment with performic acid at room temperature (Chapter 7, Section IV,D). The resultant cysteic acid groups are stable and highly polar. In extreme conditions of heat or alkalinity disulfide bonds can be broken by dissolved oxygen alone, but unreliably and with additional damage to the protein.

Albumin molecules with all disulfide bonds broken behave hydrodynamically as long strings, about 2140 Å in length, with completely random structure (Stauff and Jaenicke, 1961). The intrinsic viscosity rises from 0.1 to 0.35 (Hunter and McDuffie, 1959). All of the tyrosine residues are exposed to titration (Eisenberg and Edsall, 1963). In the common laboratory procedure of gel electrophoresis in the presence of SDS, reduced albumin migrates slightly more slowly than nonreduced albumin, evidence of the extended configuration and larger Stokes radius.

b. Reoxidation and Refolding in Vitro. Completely reduced albumin can regain an apparently native configuration after gentle reoxidation of its thiols by dissolved oxygen. Like other single-chain proteins, the information governing the eventual folding to a minimum-energy, biologically active protein is contained in the sequence of its amino acids (Anfinsen and Haber, 1961). The *mechanism* of this folding is still under study; the process is generally considered to be one of sifting by trial and error through multiple near-minimal energy configurations, beginning with local forces, to find the most stable arrangement. The folding does not require disulfide bonding; removal of an S–S bond from a simple protein by directed mutation does not affect its basic folding pattern (Laminet and Plückthun, 1989). The driving forces affecting the unfolded protein are strongly influenced by the surrounding solvent medium.

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It will be seen in this section that, even though a native configuration with all disulfide bonds complete can be achieved *in vitro*, the optimal conditions are not those that occur within the liver cell where albumin is manufactured (Chapter 5, Section I,D,2). Hence the *in vitro* simulations are probably more an exercise in protein chemistry than a model of *in vivo* events. They can enlighten us on the energetics of the albumin conformation and show some possible pathways of folding, but may not represent the much more rapid mechanism of folding during biosynthesis.

When the denaturant and the reductant are removed abruptly by dilution, as has been the usual practice, the protein must be very dilute to avoid polymerization through *inter*molecular bonding. Conditions found effective for refolding are 1–2 μ*M* albumin, pH 8.0, 0.1 *M* Tris-Cl buffer, 1 m*M* EDTA, 1 m*M* reduced glutathione, and 0.1 m*M* oxidized glutathione, at room temperature (20–25°) (Johanson *et al.*, 1981). The reduced/oxidized glutathione pair is more effective than a cysteamine/cystamine pair, or than dithiothreitol or thioglycolic acid alone. Addition of the microsomal enzyme, protein disulfide isomerase, speeds the reaction but is not essential (Teale and Benjamin, 1977). A pH of 8 is more effective than pH 7 or even pH 7.5; a temperature of 20° is more effective than 37° (Damodaran, 1986). Only BSA has been studied, perhaps because BSA is available more economically and in purer form than HSA, and its fragments are easier to prepare.

The optimal temperature for *in vitro* folding of 20° is apparently a compromise between rate of molecular motion consistent with intramolecular rather than intermolecular S–S bonding. The optimal pH of 8.0 shows the importance of SH ionization to S^- for S–S interchange. *In vivo*, where temperature is 37° and pH about 7.4, other factors must assume greater importance, such as initiation of folding before the nascent molecule is complete and facilitation of S–S bond formation by protein disulfide isomerase action.

Even at 1–2 μ*M* about half of the albumin polymerizes. At 9 μ*M* oligomers form rapidly but convert slowly to monomer (Wichman *et al.*, 1977). Concentrations as low as 0.5 μ*M* (≈30 μg/mL) have been employed (Chavez and Benjamin, 1978); inherent dangers in loss of protein to the surfaces of containers, or in the concentrating required prior to subsequent assays, set a lower limit.

Under these conditions, and with a protein as large as albumin, significant regain of native properties *in vitro* requires several hours (Fig. 2-15). The disappearance of thiol groups on the albumin, however, occurs rapidly, being 90% complete by 1 h. A possible explanation is that mixed disulfides are transiently formed and are displaced in favor of the proper intramolecular disulfide bonds in a few hours.

Regain of tertiary structure as measured by ORD or CD at 20° is complete in 8 to 24 h; the product is native as judged by solubility at pH 3 in 3 *M* KCl, by sedimentation velocity in the ultracentrifuge, and by tryptophan emission when

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stimulated at 280 nm (Andersson, 1969). Binding of fluornphores such as ANS or fluorescein shows a normal fluorescent energy yield. Binding of antibodies reaches a plateau of 80% of normal by 8 h, and of bilirubin, 75%, and long-chain fatty acids, 50%, at 24 h (Teale and Benjamin, 1977). If the refolded monomeric albumin is separated from the approximately 50% of polymer, binding of these ligands is found to be entirely restored in the monomer (Johanson *et al.*, 1981). The polymeric forms exhibit little or no fatty acid binding, about 50% of normal bilirubin binding, and 75% of antibody binding.

A more gentle approach of changing conditions, i.e., removing the chaotrope and the reductant gradually by dialysis, rather than abruptly by dilution, has been reported to give yields of monomer as high as 94% (Burton *et al.*, 1989). In these experiments the optimal conditinns were pH 10, 1 m*M* EDTA, HSA as concentrated as 5 mg/L, and sodium palmitate 20 μ*M*. This approach is obviously worthy of further study.

Native fragments of albumin refold faster and more completely than does the whole molecule (Fig. 2-15). BSA fragment 378–583 (domain III) regains

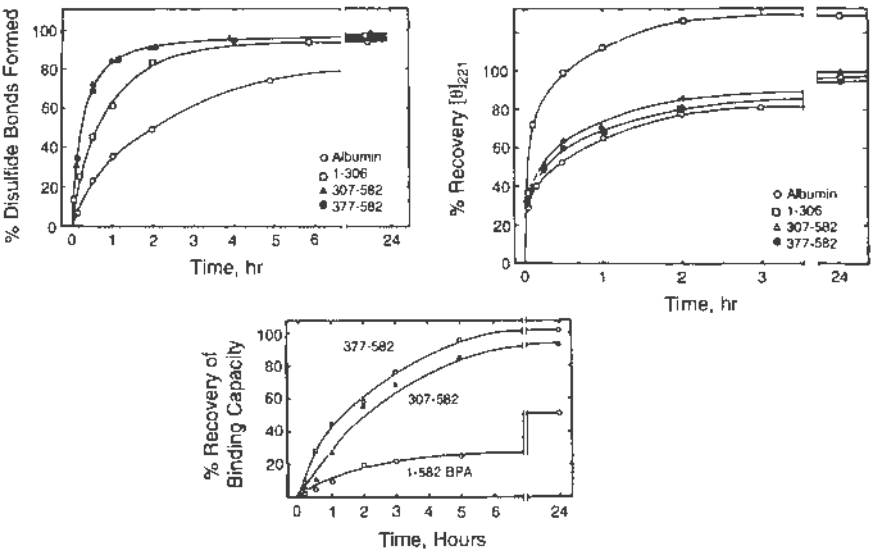


Fig. 2-15. Refolding of reduced BSA and some of its fragments. Upper left: Appearance of protein S–S bonds with time. Upper right: Return of mean residue ellipticity. Bottom: Regeneration of palmitate-binding capacity. Residue numbers 306 and higher should be increased by 1. From Johansen *et al.* (1981) by permission of *The Journal of Biological Chemistry*.

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complete palmitate-binding ability in 5 h, and fragment 308–583 in about 7 h. Return of antibody binding is similar—the smaller the fragment the faster and more complete the restoration. This finding is not surprising considering that the fragments are smaller than the whole, and that the serial arrangement of native albumin S–S bonds allows relatively independent folding.

The position of a fragment within the BSA molecule affects its rate of refolding. Fragments comprising B subdomains fold faster than do A subdomains. Among the B subdomains, loop 3 folds faster than loop 9, which is faster than loop 6 (Teale and Benjamin, 1977); return of antibody binding is stronger at the amino-terminal region than at the carboxyl-terminal one (Johanson *et al.*, 1981).

The autonomy of folding by isolated fragments favors the concept that refolding of the whole molecule begins at several nucleation sites (Wetlaufer, 1981). These would constitute the B subdomains (loops 3, 6, 9) of each domain, probably beginning with the amino-terminal domain (domain I). The sigmoidal shape of the return of binding of ANS (Damodaran, 1986) suggests an autocatalytic process, which begins slowly, accelerates, and then reaches completion more slowly as mismatched disulfide bonds are shuffled through disulfide interchange to the native, minimum-energy configuration, termed by Seckler and Jaenicke (1992) the "kinetically accessible minimum of free energy." The thiol group that remains reduced in the mature albumin molecule, CySH-34, apparently does not take part in the disulfide shuffling, perhaps owing to its remoteness from any S–S bond in the tertiary structure (Fig. 2-7); alkylation of CySH-34 prior to reduction of the albumin was without effect on the reshuffling kinetics (Johanson *et al.*, 1981).

Hydrophobic forces are critical to the regain of conformation, according to a study by Damarodan (1987). Inclusion in the refolding medium of chaotropic ions such as perchlorate or thiocyanate, 0.2 *M*, which destabilize hydrophobic forces, decreases both the rate and extent of refolding. Urea, which weakens hydrogen bonds and hydrophobic forces, is *stimulatory* up to about 2 *M* and inhibitory above that level. Its action at 1–2 *M* might be considered as a lowering of the energy barriers between various near-minima, allowing more facile testing of diverse configurations. On the other hand, the presence of sodium chloride or bromide increased both the rate and extent of folding; the maximal effect was seen at about 0.2 *M*, the concentration at which electrostatic forces in proteins are effectively neutralized. Hence ionic forces appear not to be required, and their presence may even inhibit rapid testing of alternative structural arrangements.

An intermediate, partially folded "molten globule" state can be observed if HSA is reduced with 0.02 *M* dithiothreitol at pH 9.2 without urea, or if the 8 *M* urea used as a denaturant is removed but the reductant is retained (Lee and Hirose, 1992). This form has about half of the helical content of native albumin,

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and is intermediate in size; Stokes radius is 34, 44, and 77 Å for native, intermediate, and denatured forms, respectively. The partial folding favors the regain of complete tertiary structure, because the "molten globule" completes its folding to the native S–S bond configuration twice as fast as does the reduced and denatured form. The molten globule is believed to be the result of the rapid burial of most hydrophobic surfaces.

Ligands, fatty acids (Andersson, 1969), ANS (Damodaran, 1986), or antibodies to specific fragments of BSA (Chavez and Benjamin, 1978) can increase both the extent and rate of folding of BSA. The effect is considered an illustration of "seeding" to generate nucleation centers, and is seen as well with refolding of enzymes in the presence of substrate.

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3

Ligand Binding by Albumin

Among its fellow proteins albumin is best known for its ability to bind smaller molecules of many types. This willingness to take on a varied cargo causes albumin to be likened to a sponge or to a "tramp steamer" of the circulation. The flexibility of the albumin structure adapts it readily to ligands, and its three-domain design provides a variety of sites. Literature on ligand binding by albumin from protein chemists, cell biologists, nutritionists, pharmacologists, and clinicians continues to grow and thus here only highlights and conclusions are presented. Some review articles are those of Bennhold (1961), Spector (1975, 1986), Brown and Shockley (1982), Honoré (1990), and Kragh-Hansen (1990).

Albumin interacts with a broad spectrum of compounds. Most strongly bound are hydrophobic organic anions of medium size, 100 to 600 Da—long-chain fatty acids, hematin, and bilirubin. Smaller and less hydrophobic compounds such as tryptophan and ascorbic acid are held less strongly, but their binding can still be highly specific; affinity for the L chiral form of tryptophan exceeds that for the D form by 100-fold. Table 3-1 lists examples of endogenous compounds bound by albumin. For many of these, albumin provides a depot so they will be available in quantities well beyond their solubility in plasma; in other cases it renders potential toxins harmless and transports them to disposal sites; some ligands it holds in a strained orientation, which promotes a metabolic alteration.

Although for many years ligand binding could be observed only as in a black box by measuring affinity and competition among ligands, in the past two decades mapping of sites to regions of the molecule and identification of residues forming binding sites have been made possible by specific techniques:

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TABLE 3-1
Some Groups of Endogenous Substances That Bind to Albumin

Compound	Association constant, K_A (M^{-1})	n	Reference
Long-chain fatty acids[a]	(1–69) × 10^7	1	Richieri *et al.* (1993)
Eicosanoids (PGE_1)	7 × 10^4	2	Unger (1972)
Bile acids[b]	(3–200) × 10^3	3	Roda *et al.* (1982)
Steroids			
Cortisol[c]	5 × 10^3	2	Yates and Urquhart (1962)
Progesterone[c]	3.6 × 10^5	1	Ramsey and Westphal (1978)
Testosterone[c]	2.4 × 10^4	1	Pearlman and Crepy (1967)
Aldosterone	3.2 × 10^3	1	Richardson *et al.* (1977)
Bilirubin	9.5 × 10^7	1	Brodersen (1982)
Hematin	1.1 × 10^8	1	Adams and Berman (1980)
L-Thyroxine	1.6 × 10^9	1	Kragh-Hansen (1981)
L-Tryptophan	1.0 × 10^4	1	McMenamy and Oncley (1958)
25-OH-Vitamin D_3	6 × 10^5	1	Bikle *et al.* (1986)
1,25-$(OH)_2$-Vitamin D_3	5 × 10^4	1	Bikle *et al.* (1986)
Aquocobalamin	2 × 10^7	1	Lien and Wood (1972)
Folate	9 × 10^2		Soliman and Olesen (1976)
Ascorbate	3.5 × 10^4	0.1	Molloy and Wilson (1980)
Copper(II)	1.5 × 10^{16}	1	Masuoka *et al.* (1993)
Zinc(II)	3.4 × 10^7	1	Masuoka *et al.* (1993)
Calcium	15.1 × 10^2	1	Kragh-Hansen and Vorum (1993)
	6.5 × 10^2	3	
Magnesium	1 × 10^2	12	Pedersen (1972a)
Chloride	7.2 × 10^2	1	Scatchard and Yap (1964)
	6.1 × 10^1	4	

[a] See Table 3-2.
[b] See Table 3-4.
[c] With defatted HSA.

DNA sequencing, fluorescence energy estimates of intramolecular distances, affinity labeling, X-ray diffraction, and isolation of functional fragments. Hence, it seems helpful to present first an intentionally simplified picture of the location of binding sites or regions as it can be derived currently (Fig. 3-1). Although open to criticism, it offers a model on which to attempt to understand the prolific binding data in the literature.

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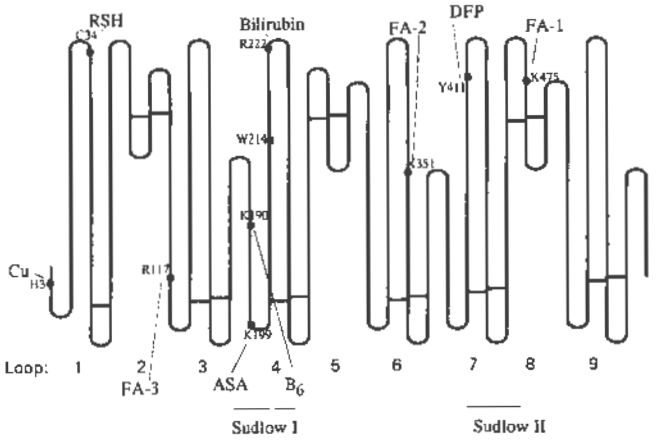


Fig. 3-1. Schematic of binding site locations on HSA. FA, Long chain fatty acids; ASA, acetylsalicylate; B_6, pyridoxal 5'-phosphate; RSH, mixed disulfides; DFP, diisopropylfluorophosphate.

Long-chain fatty acids are bound in about six sites; the three strongest of these are in different domains: (1) loops 8–9, involving Lys-475; (2) loop 6, involving Lys-351; and (3) loop 3, involving Arg-117. The weaker sites have not been identified but may include the two regions described in the next paragraph.

Salicylate, some sulfonamides, and other drugs assigned by Sudlow *et al.* (1975, 1976) to "Site I" bind in subdomain IIA, loops 4–5, involving Lys-199 and Arg-222. The bilirubin site overlaps this locus in some manner. The site for hematin does not compete for this site, but has been suggested to lie somewhere in loops 3–4. Tryptophan, thyroxine, octanoate, and drugs binding at Sudlow's Site II, often aromatic in nature, bind to subdomain IIIA, loops 7–8; this site, centered around Tyr-411, can also act catalytically to hydrolyze various esters.

Two heavy metals, Cu(II) and Ni(II), bind to the N terminus provided that the third amino acid residue is a histidine. Sulfhydryl compounds and certain oxidants bind covalently to the thiol of Cys-34. Subsequent sections will characterize the binding of these classes of ligands; of interest are affinity constants, competition for sites, distribution of the same ligand among multiple sites, effects of binding on the albumin molecule itself (allosteric conformational changes), and on–off rates in relation to delivery of a ligand to the site of its metabolism.

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I. ANIONIC AND NEUTRAL LIGANDS

The broad group of substances that might be termed endogenous or physiological anions are the most important cargo (Table 3-1). These generally bind in a hydrophobic pocket that is adaptable to the ligand, with its negative charge matched in a salt bond by the positive charge of a nearby lysyl or arginyl residue. The long-chain fatty acids are the best-known and most characteristic of these substances.

A. Long-Chain Fatty Acids

The long-chain fatty acids, oleic (C18:1), palmitic (C16:0), linoleic (C18:2), stearic (C18:0), arachidonic (C20:4), and palmitoleic (C16:1), are crucial intermediates in lipid metabolism. (C16 refers to the number of carbon atoms in the chain and the number following refers to the number of double bonds.) Typically they circulate in plasma at a total concentration just under 1 m*M*, distributed in the above order (Saifer and Goldman, 1961), and have a turnover time of about 2 min. Yet less than 0.1% of them are really "free fatty acids" in the sense of being free in the plasma. Nonesterified fatty acids is a better term. The solubility of monomeric palmitate at pH 7.4, for example, is less than 0.1 n*M*: aggregation of like molecules into micelles brings the unbound fatty acid concentration to about 10^{-4} m*M* (Vorum *et al.*, 1992). The difference, over 99.9% of the total, is transported on albumin and loaded and off-loaded with amazing speed.

A further note about terminology may be useful at this point. First, the fatty acids, having pK_A values of about 4.8, are not in the acid form at pH 7 but are soaps or the salts of fatty acids, $RCOO^-$, and properly should be called palmitate, oleate, etc. But the "acid" usage is so well entrenched that the author chooses not to combat it and will use either, *e.g.*, palmitic acid or "palmitate" interchangeably to mean the anionic salt, palmitate. Second, "long-chain" fatty acids will mean those of C_{16}–C_{20}, the ones that are highly insoluble and are important in the body. These have binding characteristics distinct from the "medium-chain" fatty acids (MCFA), C_6–C_{14}, which are much more soluble but are usually barely detectable outside of cells. The MCFA may bind to LCFA sites when these are available and when MCFAs are present in excess, but more often compete with smaller hydrophobic ligands for sites described in Sections I,A,4 and I,D below. Because they are rarely measurable in plasma, their binding is chiefly of academic or practical interest.

The Scatchard plot of binding of palmitate to BSA, Fig. 3-2 A, is resolvable into a series of about six sites of decreasing affinity. Because the total concentration of LCFAs is just below 1 m*M* in plasma, and that of albumin is about

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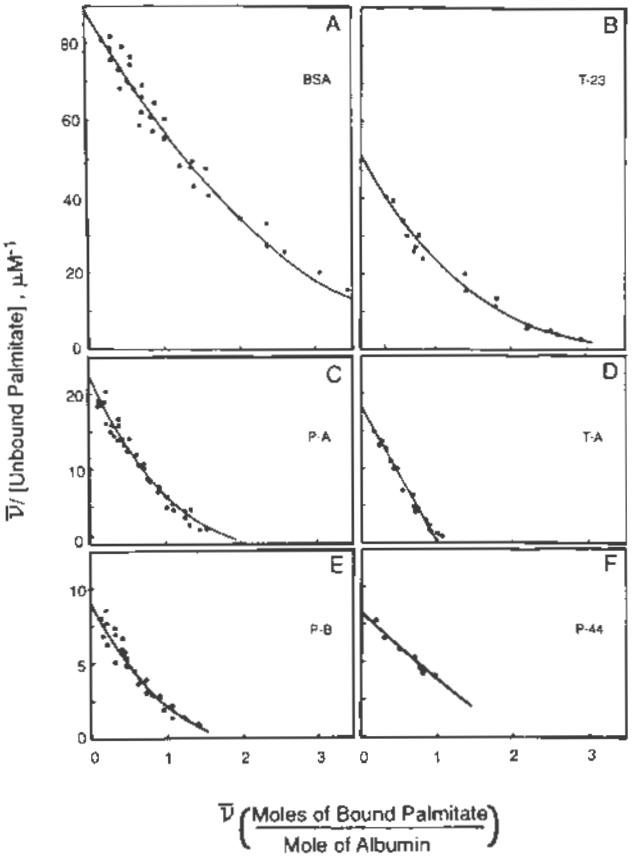


Fig. 3-2. Scatchard plots of binding of palmitate to (A) BSA and five of its fragments (see Table 2-2). Curve D (T-A) is its isolated domain III. Solid lines represent the binding curve calculated from K_A values of 34, 8.1, and 3.0 μM^{-1} for the first three sites of BSA and 18 and <0.2 μM^{-1} for domain III. Reprinted with permission from Reed *et al.* (1975a). Copyright 1975 American Chemical Society.

0.6 m*M*, circulating albumin typically carries 1 to 2 LCFA molecules; this number rises to about 4 LCFA/HSA after strenuous exercise or other adrenergic stimulation. The maximum $\bar{\nu}$ observed in the circulation, during heparin therapy, is about six; this is also the maximal loading achievable *in vitro*, following which excess fatty acids appear as a faint opalescence of the solution. Of the six potential sites, only three have been tentatively assigned locations (Fig. 3-1).

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1. Location of Binding Sites

a. Primary LCFA Site. The strongest LCFA site was first localized to domain III by the isolation of this domain from BSA, residues 377–583, T-A (Table 2-2). The fragment was prepared by allowing defatted bovine albumin to bind to a column of palmitate immobilized on agarose through its carboxyl group; the bound albumin was then digested exhaustively with trypsin and the released products washed away. The remaining material, chiefly T-A, could be released by strenuous conditions such as 0.05 *M* NaOH/50% ethanol, which removed the fragment from its association with the palmitate. Its structure appeared native by CD and its Scatchard plot (Fig. 3-2D) shows the presence of a single, strong binding site for LCFAs. Carter (1994) located the Lys^{376}-Pro^{377} tryptic cleavage locus on an exposed side of the long helix connecting domains II and III in native albumin. Lack of even a "nick" in the peptide chain of T-A after the rigorous tryptic treatment testifies to the compact structure assumed by the C-terminal domain when it binds an LCFA.

In 1986 Reed used affinity labeling, activating the carboxyl of palmitate by Woodward's reagent K, coupling it to bovine albumin, and then isolating small peptides containing the radioactive label. Of the 80% of label recovered, 45% was attached by an amide bond to Lys-474. This is consistent with the primary LCFA site involving loop 8, the middle loop of domain III. In HSA a basic amino acid homologous to BSA Lys-474 is Lys-475 or Arg-473 in helix h5. The affinity label would detect only a lysine, and chemical shift changes with pH were characteristic of involvement of ε-amino groups (Cistola *et al.*, 1987b). The nearby arginine group is another possibility for matching the LCFA anion. Modification of guanidinyl groups has been shown to interfere with fatty acid binding (Jonas and Weber, 1971).

A subsequent NMR study using bovine albumin fragments with ^{13}COOH-oleate (Hamilton *et al.*, 1991) showed evidence for two strong sites in domain III, one strong site in domain I, and weaker ones in domain II. That the single recognized site in domain III might actually bind a *pair* of LCFA molecules was proposed from study of ^{1}H NMR of histidine C-2 proton signals (Oida, 1986a) as well as from an earlier spectral study of polyene fatty acid binding (Berde *et al.*, 1979). This raises the question whether the binding site is a pocket or a tunnel. If it is a pocket, it would seem that the fatty acids would have to be in a tail-to-tail (parallel) orientation to avoid the problem of inserting a charged COO^- group deep into the albumin molecule. But the finding that the binding of a double-ended LCFA such as ω-octadecanedioic acid to the first site ($K_A = 0.5 \times 10^6$ M^{-1}) is blocked by oleate could mean that these two LFCAs compete for the same site, and that the negatively charged group can be oriented to either end of a hydrophobic tunnel or channel (Tonsgard and Meredith, 1991).

Data from electron spin resonance (ESR) studies using nitroxyl reporter groups also appear to support the tunnel concept. Two stearic acid molecules

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containing a nitroxide at position 10 bind to HSA significantly less strongly than two molecules with the nitroxide at position 5 (Berde *et al.*, 1979), implying that the center of the fatty acid is normally more tightly bound than the ends. Other ESR studies are in agreement that the middle of the bound carbon chain is more restricted than its ends (Hsia *et al.*, 1984; Perkins *et al.*, 1982). Ge *et al.* (1990), using stearic acid with doxyl labels at different positions along its chain, propose that the tightest restriction in the binding channel is between C-5 and C-13–15, and that the channel is 11 ± 1 Å long.

Such channels, lined with helical segments, were demonstrated by Brown and Shockley (1982) from model building and were confirmed by the X-ray data showing helix h5 antiparallel to helix h6 in loop 8, and helix h8 antiparallel to h9 in adjacent loop 9. Such a helix-lined pocket is distinctly different from the β barrel that has been identified as the LCFA site in the fatty-acid-binding proteins of tissues, the barrel being formed by 10 orthogonal antiparallel ββ sheets (Banaszak *et al.*, 1994).

b. Secondary LCFA Sites. In Reed's affinity labeling experiments cited above, another 45% of the palmitoyl label was found with BSA Lys-350 in loop 6 and 10% with Lys-116, between loops 2 and 3. Lys-350 was also implicated as the site to which attachment of trinitrobenzenesulfonate to BSA was blocked by palmitate in an earlier study (Andersson *et al.*, 1971) (assuming that the peptide the authors reported as LAEKY was, in one-letter code, actually LAKEY). This point of attachment in loop 6 is in agreement with the presence of an LCFA site in subdomain IIB, indicated from studies with isolated fragments such as P-A, loops 6–9 (Table 2-2); Scatchard plots (Reed *et al.*, 1975a) rated this as the second-strongest site. BSA Lys-350 is homologous to Lys-351 in HSA helix h9. A helical pocket could embrace loop 6 and the ascending limb of loop 7, between domains II and III; helical segments involved would be helices h8, h9, and h10 of domain II. Brown and Shockley (1982) also supported the presence of a LCFA site in subdomain IIB.

c. Tertiary LCFA Sites. Evidence for a third, somewhat weaker site suggested by ^{13}C NMR is the 10% of palmitate label found on BSA Lys-116, homologous to HSA Arg-117, and the observation of Brown and Shockley that reaction of HSA His-145 with *N*-dansylaziridine was blocked by the presence of stearate, palmitate, or oleate (Brown and Shockley, 1982). This region of loop 3 has long helical stretches h8 and h9 with adjacent shorter helices in loop 2, h5 and h6. Thus, as indicated in Fig. 3-1, the three strongest LCFA sites can be mapped to the C-terminal (subdomain B) portions of domains I, II, and III in increasing order of affinity. Whether all three sites may be channels rather than pockets as proposed for the primary site cannot be judged with the evidence presently available.

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2. Thermodynamics and Mechanism of Binding

The extremely low solubility of LCFAs and their inability to pass through dialysis membranes make it impossible to study LCFA binding by means in general use for smaller, more soluble molecules. Various devices have been employed to surmount this difficulty.

Binding studies usually start with fat-free albumin. In the most common defatting procedure the albumin solution is brought to pH 3, where the fatty acids are released (and, if their quantity is high, can be seen as a faint opalescence) and removed by adsorption onto charcoal (Chen, 1967) or onto a hydrophobic resin surface (Scheider and Fuller, 1970). Use of H_2SO_4 to avoid the presence of chloride, which can affect fatty acid binding, has been recommended. Although pH 3 is not severe treatment for albumin, Scheider later developed a procedure to remove fatty acids at pH 7.4 by incubating the albumin with mouse adipose tissue; decrease from 0.22 to 0.05 M/M was reported (Scheider *et al.*, 1976). Bacterial growth is a concern, and there seems to be a risk of accumulating unwanted cell metabolites on the albumin.

Pedersen *et al.* (1990) have measured the *rate* of exchange of radiolabeled fatty acids through a dialysis membrane between two identical solutions of serum albumin or plasma specimens. The problems remain of lack of free passage of a long, fatty molecule such as palmitate through the pores of a membrane, and of its tendency to stick to the membrane. The method works well with MCFA (C_{12}–C_{14}), but again only indirect estimates can be obtained for the LCFA (C_{16}–C_{20}).

Reed *et al.*, (1975b) developed the technique of measuring exchange of a ligand between an albumin solution and BSA immobilized on agarose. The coupling of the BSA did not appear to affect its binding ability for palmitate or bilirubin. This method is simple in application, although it does not have an absolute K_A, but rather compares the binding of a test albumin with that of an albumin of known K_A used as a calibrator.

The late D.S. Goodman (1958) added an intermediate solvent such as heptane, which is immiscible with an aqueous albumin solution, in order to estimate the concentration of free LCFA, e.g., palmitate, in equilibrium with the palmitate bound to the albumin [see also Spector (1986) for details]. The partition ratio of palmitate concentration in the heptane and in the protein-free aqueous solution, about 500:1, was established in separate experiments. As pointed out by Spector *et al.* (1969), a pitfall is ignorance of the extent of aggregation of the aqueous palmitate. Calculations showed that a moderate degree of dimerization of the unbound palmitate would cause about a fourfold increase in apparent association constants for palmitate. A second pitfall is traces of labeled hydrophobic impurities in the radiolabeled LCFA (Burczynski *et al.*, 1993).

The obstacle of measuring the true free fatty acid concentrations appears to have been overcome by the use of a "reporter" compound that responds to the

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presence of LCFA by a change in fluorescence emission wavelength (Richieri *et al.*, 1993). The reporter is recombinant mouse intestinal fatty-acid-binding protein modified by attachment of acrylodan (6-acryloyl-2-dimethylaminonaphthalene) at an ϵ-amino group.

Binding isotherms obtained by this technique in a buffer of pH 7.4 containing HSA and NaCl at 37° are seen in Fig. 3-3. The inset shows the free LCFA levels at the normal LCFA/albumin ratios in plasma, 1–2; these are less than 10 n*M* for stearate, palmitate, and oleate, rising to about 20 n*M* for linoleate and arachidonate and 50 n*M* for linolenate. The free LCFA levels rise smoothly with $\bar{\nu}$ at an accelerating rate until at $\bar{\nu} > 5$ the effect of the albumin binding becomes minimal. The data of Fig. 3-3 were obtained with [HSA] $\approx$ 6 μM, only about 1% of that in plasma, but no change was seen with levels between 1 and 10 μM [HSA] at $\bar{\nu} < 3$, and it seems likely that the same relationships would apply at the HSA concentration in plasma ($\approx$600 μM).

Interpretation of these curves, like the Scatchard plot of Fig. 3-2, is ambivalent. One procedure for analyzing binding isotherms calculates several classes of strong and weak sites. With this approach Goodman (1958) proposed that, for oleate, there are two strong sites and four weaker ones, an approach still tenable.

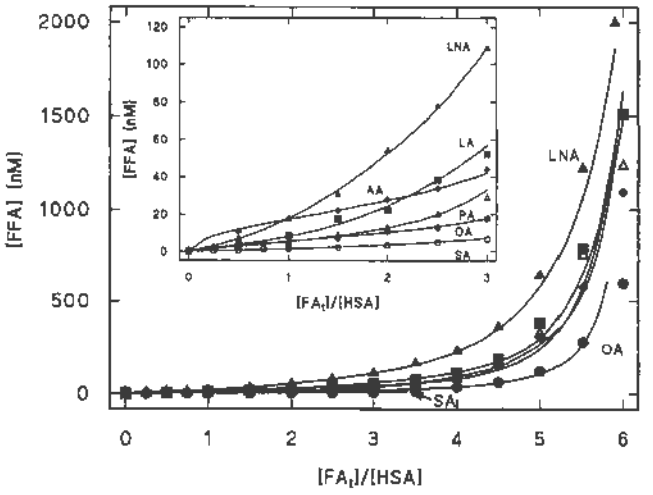


Fig. 3-3. Binding isotherm for long-chain fatty acids. SA, Stearic acid; OA, oleic acid; PA, palmitic acid; LA, linoleic acid; LNA, linolenic acid; AA, arachidonic acid; FFA, free fatty acid. Reprinted with permisson from Richieri *et al.* (1993). Copyright 1993 American Chemical Society.

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The stepwise approach, likewise an oversimplification, analyzes the binding as a series of stepwise equilibrium reactions, assuming filling of strong sites followed serially by weaker and weaker sites (Ashbrook *et al.*, 1975).

Association constants obtained by applying the stepwise analysis to data of Fig. 3-3 for HSA are shown in Table 3-2. Considering the first sites only, the saturated LCFA stearate and palmitate bind most strongly, then linoleate, oleate, linolenate, and arachidonate, in that order. A smooth decrease in the affinities of the saturated LCFA series, $C_{18} > C_{16} > C_{14}$, etc. (Spector, 1975), is believed to be related to the decreased opportunity for hydrophobic bonding. The weaker binding by unsaturated LCFAs may reflect their structural bends at *cis*-bonds, which influence the fit to a binding site.

LCFAs are generally strongly bound by albumins regardless of species; differences are small and vary with the fatty acid. BSA (Table 3-2), studied similarly to HSA, binds palmitate slightly less strongly than does HSA; it binds the first oleate more strongly than does HSA but the next three oleates less so. Linoleate and linolenate bind less well to BSA but arachidonate binds more strongly. Affinities for mouse SA are generally lower than for HSA and BSA (Richieri *et al.*, 1993), whereas those for rabbit albumin are intermediate (Spector *et al.*, 1969).

When several binding sites of different affinities are available for a ligand such as a LCFA, they do not strictly fill in decreasing order of affinity. Rather,

TABLE 3-2
Binding of Long-Chain Fatty Acids and Other Lipids[a]

	HSA		BSA	
Compound	K_A ($10^7 \times M^{-1}$)	n	K_A ($10^7 \times M^{-1}$)	n
Stearate	68.9	1	—	—
Palmitate	14.5	1	12.2	1
Linoleate	13.5	1	4.5	1
Oleate(site 1)	11.8	1	12.7	1
Oleate(site 2)	11.8	1	10.0	1
Oleate(site 3)	8.0	1	4.0	1
Oleate(site 4)	5.5	1	3.0	1
Linolenate	9.5	1	2.7	1
Arachidonate(site 1)	1.1	1	3.4	1
Arachidonate(site 2)	7.8	1	—	—
Lysolecithin[b]	—	—	0.004	—

[a]From Richieri *et al.* (1993).
[b]From Klopfenstein (1969).

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the less divergent the K_A values, the more widely the ligand molecules will be distributed among different sites on different protein molecules. Even when the total LCFA bound was ≤1 M/M BSA, NMR studies with ^{13}COOH-stearate showed five or more binding resonances, and implied that the ligand is distrib uted among three different sites (Cistola *et al.*, 1987a). Spector and Fletcher (1978) calculated the distribution of oleate among HSA molecules, a sample of which is given in Table 3-3. It seems surprising that, at an average $\bar{\nu}$ of 1, 31% of the albumin molecules have no oleate, 22% have 2 oleates, and only 43% have the average of 1 oleate. At an average of 2 M/M, only 40% have the average number and a total of 28% have 3 or more ligands. At mole ratios greater than 6, free soaps were detected in crystalline form by X-ray diffraction (Cistola *et al.*, 1987a), and as microspheres of 2000–7000 Å by electron microscopy (Brodersen *et al.*, 1989).

These oleates are constantly exchanging among sites on albumin molecules via the free state. As noted in Chapter 2 (Section II,C,2,c), when fat-free albumin molecules, which are more susceptible to heat denaturation, are removed by heat coagulation, the remaining molecules accumulate more and more oleate until the last ones to remain have about 6 M/M. A similar effect is noted on prolonged electrophoresis, as in isoelectric focusing; the free fatty acids are swept toward the anode, and accumulate on a band of fully loaded albumin molecules at about pH 4.8, leaving a band of fat-free albumin at pH about 5.8 (Gianazza *et al.*, 1984).

TABLE 3-3
Distribution of Oleates among Albumin Molecules at Different Average Loadings[a]

Number of oleates/albumin	Percent of albumin molecules with listed number of oleate molecules	
	Ave. $\bar{\nu} = 1$	Ave. $\bar{\nu} = 2$
0	31	6
1	43	26
2	22	40
3	4	20
4	0	7
5	0	1
	100	100

[a]From Spector and Fletcher (1978).

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A binding constant, K_A, is the ratio of the association and dissociation rate constants

$$\text{Alb} + \text{FA} \underset{k_d}{\overset{k_a}{\leftrightarrows}} \text{AlbFA}; \quad K_A = k_a/k_d. \tag{6}$$

Svenson *et al.* (1974) estimated the dissociation rate constant from the speed of uptake of palmitate from soluble HSA to immobilized HSA as 0.04 s^{-1} at 23°. Speeds of uptake must be much higher to yield K_A values > 10^8 M^{-1}. Scheider has approached the uptake process by measuring the dielectric increment caused by the change in shape of HSA on binding oleate under stopped-flow conditions (Scheider *et al.*, 1976). To measure uptake, he mixed HSA and oleate; to measure release, he observed the transfer of oleate from HSA to BSA.

Scheider's interpretation is that the binding occurs in two distinct steps; a rapid (<1 ms) but loose ionic attachment of the oleate at the surface of the albumin, followed by a slower (300 ms) unfolding and reclosing of a hydrophobic channel in the protein, which allows the hydrophobic end of the fatty acid access to the interior. The "breathing" of the protein is an entropy-driven process, involving access of solvent water to the interior followed by exclusion of these molecules again as the hydrocarbon chain attaches and the pocket refolds. It is essentially independent of temperature.

Thermodynamic parameters of this two-step binding are illustrated in Fig. 3-4. Scheider's data show overall $\Delta G = -10$, $\Delta H = -18$, and $T\Delta S = -8$ kcal mol^{-1}. Spector *et al.* (1969) found, for three primary sites, $\Delta G = -9.5$, $\Delta H = -17.7$, and $T\Delta S = -8.2$ at 37°. Aki and Yamamoto (1992) also found the binding to be entropy-controlled using flow calorimetry: ΔH −10, $T\Delta S$ decreasing from −3.5 to −0.6 with increasing chain length. The first step is diffusion controlled, and involves only a small ΔH change. The second step involves a negative entropy of activation, or unfolding. Formation of the ionic bond with the fatty acid carboxyl group is associated with an enthalpic change; as Scheider noted, this is the only true chemical bond; the remaining forces are hydrophobic attractions. Should paired oleates occupy a site, the entropic (hydrophobic) contribution would be greater.

3. Effects of LCFA Binding on Albumin Molecule

When the flexible albumin molecule closes around a long-chain fatty acid, some adjustments occur in the protein in addition to constraining the LCFA. There have been many reports of conformational and other changes in albumin brought about by MCFA, C_8–C_{14}, including crystallization of HSA with octanoate. Here, however, we will consider as far as possible only effects brought about by the first two or three ligands of LCFA, C_{16}–C_{20}, which are

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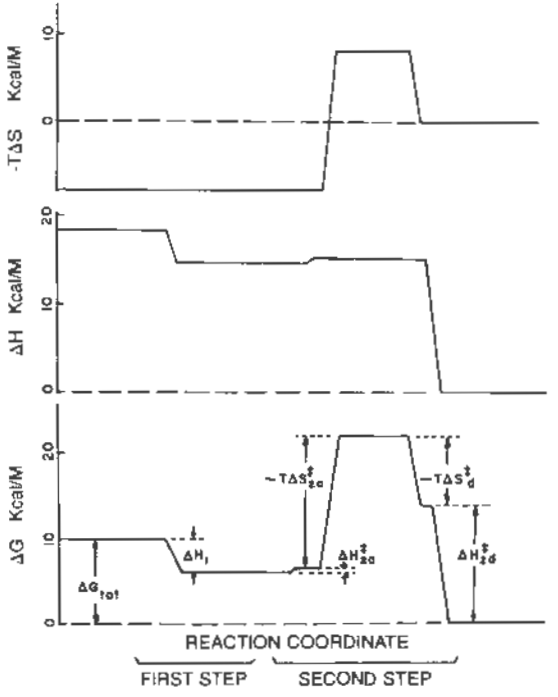


Fig. 3-4. Thermodynamics of long-chain fatty acid binding. Reproduced from Scheider (1979).

believed to exclude MCFA from their primary sites. Because of the distribution among sites (Table 3-3), an average $\bar{\nu}$ of two LFCA/SA would produce effects at each of the three LCFA sites shown in Fig. 3-1 in some of the albumin molecules in a solution.

The major effect seems to be a change in shape with an increase in stability of the albumin. With 1–2 LCFAs the shape becomes more compact (Dzhafarov, 1992) and more rounded as judged by decreased dielectric increment and by more rapid electrophoretic migration (Scheider, 1979; Soetewey *et al.*, 1972). The calculated ellipsoidal axes change from 143 × 34 Å to 128 × 41 Å.

Compared to fatty-acid-free albumin, increased stability is seen on storage, heating (Brandt and Andersson, 1976; Shrake and Ross, 1988), and proteolytic digestion. Strenuous treatment of BSA with trypsin (0.6 mg/mL) at the optimal pH of 8.8 for tryptic activity leaves domain III unscarred if the protein is bound to immobilized palmitate, but only very limited tryptic treatment [1:1000 (w/w), pH 8.15] can be used in solution without destroying such large fragments (Peters

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and Feldhoff, 1975). Other studies, although using lauric acid, have described protection from action of trypsin, chymotrypsin, and bacterial proteases (Kondo, 1962). Refolding of completely reduced HSA is facilitated by the presence of 10 μM palmitate (Andersson, 1969), presumably through stabilizing the initial folding around the hydrophobic LCFA site(s).

FTIR results suggest some disordering of the helical regions, together with some increased accessibility of histidine residues (Le Gal and Manfait, 1990). Tyrosines show decreased fluorescence and a small red spectral shift (observed with C_{12}) (Steinhardt *et al.*, 1972). The single tryptophan residue of HSA appears unaffected, but access of iodide ions that quench the fluorescence of the second tryptophan of BSA (in loop 3) is restricted, suggesting that the LCFA site in domain I has become more compact (Spector, 1975). At this same site, on the other hand, increased oxidation of the thiol of Cys-34 accompanies binding of 1–2 oleates, but not palmitate, perhaps from a peroxidative action (Noel and Hunter, 1972) or from a widening of the crevice containing the thiol (Takabayashi *et al.*, 1983). This report recalls the common finding that there is an inverse correlation between thiol content and LCFA content in albumin separated on DEAE-agarose, perhaps the result of such oxidation (see also Table 7-5 and discussion in Chapter 7). Takabayashi *et al.* also found that nonmercaptalbumin has a greater affinity for LCFA than does mercaptalbumin, either in solution or on palmitoyl-agarose.

Despite the above structural changes, cooperativity is apparently low with LCFA binding. The K_A for subsequent LCFA sites is generally smaller as $\bar{\nu}$ increases (Table 3-2). An exception may be arachidonate, which has increased affinity to its second site. This tendency is evident in the unique shape of the binding isotherm for arachidonate at $\bar{\nu} = 0$–2 (labeled AA in inset to Fig. 3-3).

4. *Binding of Related Lipids*

Aliphatic compounds lacking the carboxyl group are generally bound less tightly than are LCFAs. The glyceryl ester, monoolein, is among those studied (Arvidsson and Belfrage, 1969). Ethyl esters of LCFAs are another example; they have been detected in association with albumin in plasma of subjects following ingestion of ethanol (Doyle *et al.*, 1994).

Ultracentrifugation and gel filtration show that the lysolecithins of plasma, about 10% of plasma phospholipids, are associated with albumin rather than with lipoproteins (Switzer and Eder, 1965). They bind weakly (Table 3-1), and binding is by their hydrophobic regions, because NMR shows the polar cation to be unrestricted (Steim *et al.*, 1968). Those containing saturated LCFAs are held at a single site (Barlow and Klopfenstein, 1980); unsaturated forms show a 2:1 stoichiometry. Oleoyllysophosphatidic acid binds more strongly than the choline and ethanolamine derivatives, and because it competes with oleate apparently binds at the primary LCFA sites (Thumser *et al.*, 1994). Oleate does not inhibit

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lysophosphatidylcholine binding (Rosseneu-Motreff *et al.*, 1974), so lysolecithins and lysophosphatidylethanolamines probably do not occupy the strong LCFA sites, but compete with MCFAs.

A compound not commonly found in plasma, palmitoyl-coenzyme A, has been observed to bind to BSA. The complex is not of significance *in vivo* but can be a concern to enzyme chemists studying intracellular metabolism because of the detergent action of the free palmitoyl-CoA. Scatchard analysis indicates two primary sites, $K_A = 6 \times 10^5\ M^{-1}$, plus four weaker sites in the manner of palmitate binding but with reduced affinity (Richards *et al.*, 1990).

The complex lipid of *Salmonella* lipopolysaccharide, lipid A, by contrast, is rendered more toxic when bound to BSA or HSA (Rietschel *et al.*, 1973). Lipid A contains 3-OH myristate acylated at its OH group by C_{12}, C_{14}, and C_{16} fatty acids. The bound form has increased pyrogenicity in rabbits, apparently through wider distribution in the circulation.

Alcohols of the long-chain fatty acids do not appear to have been studied, but dodecanol, $K_A = 1.5 \times 10^5\ M^{-1}$, $n = 4$–5 (at 2° and pH 5.6), binds to BSA less strongly than its counterpart, lauric acid. The saturated hydrocarbon, octane, binds about as strongly as the homologous alcohol, octanol, $K_A = 3400\ M^{-1}$ (Ray *et al.*, 1966). NMR of lower (C_1 to C_4) alcohol binding shows a weak hydrophobic association that increases with chain length and decreases with branching (Lubas *et al.*, 1979). Long-chain ω-dicarboxylic fatty acids appear to bind first at the primary LCFA site. The affinity constant for octadecanedioic acid was reported as $5.3 \times 10^5\ M^{-1}$ (Tonsgard and Meredith, 1991).

At the other end of the scale the strongly anionic detergent, dodecyl sulfate, binds about twice as tightly as its corresponding carboxylate—8–9 sites with $K_A = 2.3 \times 10^6\ M^{-1}$ compared to $1.2 \times 10^6\ M^{-1}$ (Ray *et al.*, 1966). The highest carbon sulfonate studied appears to have been C_{14} (myristate) (Steinhardt *et al.*, 1972). SDS has been a popular compound for study of protein–ligand interactions (Jacobsen, 1977; Foster, 1960). Large amounts, $\bar{\nu} > 100$, can be bound cooperatively with opening of the native structure (Chapter 2, Section II, C,2,a): the binding is not as specific for albumin as is that of LCFAs. ^{13}C NMR indicates that the sulfate and adjacent CH_2 group are in a polar environment, whereas the remaining CH_2 groups are in a hydrophobic region and the terminal CH_3 associates with other detergent methyl groups in the vicinity (Kragh-Hansen and Riisom, 1976).

Cationic detergents bind much less strongly than do anionic ones of similar chain length. Tetradecyltrimethylammonium chloride is the longest chain species studied. Aggregate bands are seen on electrophoresis of mixtures with BSA (Aoki and Hiramatsu, 1974).

a. Eicosanoids. Although arachidonate is truly a long-chain fatty acid, other eicosanoid (C_{20}) derivatives, such as leukotrienes, prostaglandins, and

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thromboxanes, are less likely to be bound at the strong LCFA sites; however, this important class of lipids is considered here for lack of a more suitable place. Highly unsaturated and folded, they are both more hydrophilic and more compact than the LCFAs. Arachidonate exhibits an apparent cooperative mode of binding, but a weaker one than other LCFAs (Table 3-2). Even so, the binding of these eicosanoids by albumin has been found to have significant metabolic consequences (Chapter 5, Section II,B,4).

Sequestration by albumin increases the release of arachidonate from phospholipids (Dieter *et al.*, 1990), whereas it modifies the metabolic pathways of resulting eicosanoids in several ways. It favors the action of lipooxygenase more than that of cyclooxygenase in leukocytes (Broekman *et al.*, 1989). The leukotriene product, LTA_4, is stabilized by allowing its stereoisomerization to LTB_4 to proceed (Folco *et al.*, 1977).

Among the cyclized products, albumin binds the prostaglandins (PGs) A, E, F, and I weakly. The binding of PGI_2 protects it from rapid degradation and makes it more available for platelet receptors (Tsai *et al.*, 1991). Binding promotes the conversion of the endoperoxide, prostaglandin H_2 (PGH_2) to PGD_2 rather than PGE_2, in which the carbonyl and hydroxy groups on the ring are interchanged (Hamberg and Fredholm, 1976). PGD_2 conversion proceeds further to form three other products (Fitzpatrick and Wynalda, 1983). The dehydration of PGE_2 to PGA_2 is stimulated, allowing it then to isomerize to form PGB_2 (Fitzpatrick *et al.*, 1984).

The dehydration reaction affects particularly prostaglandins with a β-hydroxy ketone structure. The reaction requires strong alkali in aqueous solution, but occurs in the neutral range in the microenvironment in which these eicosanoids are held by albumin.

Some of the thromboxanes are bound and their stability modified either upward or downward. $TBxA_2$ is spared from destruction by albumin, whereas $TBxA_1$ is destroyed more rapidly (Folco *et al.*, 1977). Some of the $TBxA_2$ appears to bind covalently to HSA, as does PGI_2 (Maclouf *et al.*, 1980).

Several bits of evidence suggest that the eicosanoid binding occurs at Sudlow's Site I in subdomain IIA (Fig. 3-1). (1) The capacity of albumin to bind arachidonate is decreased by about 50% in neonates (Sadowitz *et al.*, 1987), a phenomenon seen also with bilirubin and which may be due to a compound (2-hydroxybenzoylglycine—Chapter 6, Section II,B,6,a) competing for that site. (2) Phenoxybutazone and warfarin, compounds binding at Site I, both compete with PGE_2 binding (Fitzpatrick *et al.*, 1984). (3) Studies with a fluorescent probe attached to PGI_2 indicate that it is bound only 15–18 Å from the single tryptophan of HSA, located in loop 4 (Tsai *et al.*, 1989) near Site I. Fitzpatrick, one of the leading workers in the field of eicosanoid binding, has suggested that the sequence 211–218 containing this tryptophan in HSA could provide the alkaline environment favoring dehydration (Fitzpatrick *et al.*, 1982).

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The aromatic retinoids, retinoic acyl relatives of eicosanoids, are apparently not bound to an appreciable extent by albumin in plasma, but rather are carried by lipoproteins (Carillet *et al.*, 1990). The K_A for retinoic acid for albumin has been reported as 10^5 and $10^4\ M^{-1}$ at two sites (Nerli and Pico, 1994); its presence perturbs binding of drugs at both Site I and Site II.

b. Steroid Hormones. The binding of steroid hormones by albumin is weak (Table 3-1) and there are carrier proteins for them in plasma that are more specifically designed for the task. But the huge relative excess of albumin more than compensates for its low affinity, so that most of the hormone is often carried by albumin (Watanabe *et al.*, 1991). The low affinity actually means that it is the hormone molecules carried by albumin that are the more rapidly released and delivered to tissues (see Chapter 5, Section II,B,2).

The principal steroid-specific transport proteins are transcortin (cortisol-binding globulin) and sex hormone-binding globulin (testosterone-binding globulin). Steele *et al.* (1982) showed that, as they titrated plasma with prednisolone, at concentrations up to 1 μg/L, 95% was bound by transcortin but at higher concentrations the binding became nonlinear and fell to 80% at 1.8 μg/L. Their model showed that the binding to transcortin was saturable at low hormone levels and that subsequent binding was nonsaturable and almost independent of albumin concentration at higher levels within the physiological range.

Structural requirements of the steroids for binding were described by Romeu *et al.* (1975), who measured ligand bonding by effects on the intrinsic fluorescence of the tryptophans of BSA. Steroid–albumin interaction is increased by a benzenoid A ring (estrogens), the 19β-methyl group, sulfate or carboxylate ions near C-3, acetylation at various sites, and the 17β-hydroxy group, for example. Interaction is decreased by polar (oxygen) atoms at carbons in positions 3, 6, 11, 16, and 17, acetylation at C-3, a Δ-1 conjugated double bond, and certain other features. They concluded that, with planar, nonaromatic steroids, binding to albumin chiefly involves the α (rear) surface of the B, C, and D rings and possibly the 17β side chain. Cekan *et al.* (1984) studied binding of steroid sulfates and concluded that albumin has one strong but nonspecific site, but that estrone sulfate is not bound *in vivo* owing to preferential occupancy of the site by other ligands.

A weak binding of free (nonesterified) cholesterol to albumin in the circulation has been detected (Deliconstantinos *et al.*, 1986). After injecting [^{14}C]cholesterol into rats the amount found with albumin isolated subsequently by mild procedures was estimated at 4.6 mg/100mL serum. This would correspond to 24% of the unesterified cholesterol in rat serum.

Recent evidence with large fragments suggests that testosterone and pregnenolone bind in domain II of HSA (Fischer *et al.*, 1993); Brown and Shockley (1982) tentatively proposed a site in loop 6 from model building. Cortisol does not interfere with testosterone binding (Pearlman and Crepy, 1967); thus there

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seem to be at least two steroid sites. Oleate has been reported to stimulate progesterone and testosterone binding but not that of estrogens (Watanabe *et al.*, 1990), whereas depression of progesterone binding by palmitate was observed in an earlier study (Ramsey and Westphal, 1978); a possible difference among commercial albumin preparations was suspected. More recently no effect of oleate on the unbound concentrations of cortisol, testosterone, or estradiol was found when measured in human serum (Watanabe *et al.*, 1991).

Soltys and Hsia (1978) noted competition of steroids with a spin-labeled compound purported to occupy the bilirubin site (Site I, Fig. 3-1). Morrisett *et al.* (1975) used spin-labeled derivatives of androstol, indole, and LFCAs and suggested from NMR and fluorescence findings that the sites for all three of these compounds may be identical. Observations following chemical modification and fluorescence have indicated that Trp, Tyr, Arg, and Lys residues of BSA participate in the binding site (Romeu *et al.*, 1976). Progesterone was found to accelerate tryptic cleavages of BSA and HSA, but androgens mainly inhibited and cortisol was neutral (Ryan, 1973).

c. Vitamin D. This steroid derivative is chiefly carried by the specific transport agent, DBP (Chapter 4, Section II,C). Even though its concentration in plasma betters that of DBP by 100:1, albumin carries less than 12% of 25-OH-vitamin D and only about 15% of the 1,25-$(OH)_2$ form. The K_A values for albumin are 6×10^5 and 5×10^4 M^{-1}, respectively, for the mono aud dihydroxy forms (Table 3-1), over three logs less than the affinity for DBP (Bikle *et al.*, 1986).

d. Bile Salts. Bile salts, usually called bile acids in the same loose manner that fatty acid salts are called fatty acids, are another class of steroid compounds that are bound primarily to albumin in the circulation. Again the author will bow to convention and generally refer to them as the bile acids.

The affinity of HSA for bile acids is about two logs less than for the LCFAs (Table 3-4). Predictably, affinity is highest for the most hydrophobic form, lithocholate; it deceases about 75% for the three forms of deoxycholate, and almost 100-fold for the dihydroxy form, cholate. The data shown in Table 3-4 were obtained by equilibrium dialysis and represent the first class of sites analyzed with $n = 3$; data from another such study were analyzed with $n = 1$ and show $K_A = 500 \times 10^4$ and 25×10^4 M^{-1} for lithocholate and chenodeoxycholate, respectively. Conjugation with highly polar glycine or taurine depresses the affinity only slightly—20 to 30% (Table 3-4).

Studies with probes to localize the binding sites for bile salts on HSA found only dansylsarcosine, which binds at Sudlow's Site II in subdomain IIIA (Fig. 3-1) and oleate at high concentration (Beckett *et al.*, 1981) to compete. From the nature of the competition Takikawa *et al.* (1987) concluded that the primary site

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TABLE 3-4
Binding of Bile Acids to Albumin[a]

Compound	Association constant, K_a ($10^3 \times M^{-1}$)	n
Lithocholate	20.0	3
Chenodeoxycholate	5.5	3
Deoxycholate	4.0	2
Ursodeoxycholate	3.8	3
Cholate	0.33	3
Glycolithocholate	19.6	3
Glycochenodeoxycholate	4.9	2
Taurochenodeoxycholate	4.5	3
Glycodeoxycholate	3.5	3
Glycocholate	0.26	3
Taurocholate	0.18	3

[a]From Roda *et al.* (1982).

for bile acids on albumin is unique, not previously recognized, but that the secondary locus may be Site I. Bowmer *et al.* (1985) found competition from warfarin and sulfa derivatives, which are considered to be Site-I ligands. Fluorescent energy transfer data, however, prompted Farruggia and Pico (1993) to propose that bile acids bind to Site II.

With BSA, fluorescent studies have implicated the region near the tryptophans. The hydroxy bile acids quench the tryptophanyl fluorescence about 50%, but the oxidized, keto forms quench completely. A similar effect was seen with the fluorescence from ANS, which is energized from the tryptophans (Pico and Houssier, 1989). The implication is that one of the tryptophan residues is accessible to the hydroxy forms but both are accessible to the keto forms.

The location of the bile acid site(s) is obviously unclear. The thermodynamics of their binding (Scagnolari *et al.*, 1984) shows large entropy changes consistent with a largely hydrophobic interaction, a concept supported, perhaps, by the very minor effect of conjugation with the hydrophilic compounds glycine and taurine.

In plasma, albumin accounts for 88% of the bound bile acids, the remainder being linked to lipoproteins of all classes (Malavolti *et al.*, 1989). During clinical cholestasis, when the circulating bile salt levels climb markedly, it is the binding to lipoproteins, especially high-density forms, that increases rather than that to albumin (Buscher *et al.*, 1987). Part of this effect has been attributed to

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the competition by the concomitant elevation of bilirubin, again giving a suggestion that bile salts occupy Site I. The albumin in lymph is free of bile salts, a finding ascribed to competition from the elevated LCFA transported in lymph (Beckett *et al.*, 1981).

B. Bilirubin

The single most well-studied ligand of albumin is undoubtedly bilirubin. The binding mechanism of this complex compound continues to intrigue protein chemists; it detoxifies its antimitochondrial action, promotes its antioxidant effects, and aids in its destruction by light *in vivo*. Brodersen's laboratory in Aarhus has been particularly active in this field, and his thorough review (1980) is recommended for detailed information.

Bilirubin IXα ZZ, the prevalent form in the body, behaves in plasma as a hydrophobic substance requiring albumin to make it soluble. This behavior is strange considering the presence of two hydrophilic carboxylates and two lactam rings in its four-ring structure (Fig. 3-5A). The reason for its low solubility in aqueous solution is that the four pyrrole rings of bilirubin IXα curl together into a compact form, held by intramolecular hydrogen bonds between the two propionate carboxyls and the pyrrole nitrogens of the opposing pyrrole pairs, and between propionic acid protons and carbonyls of rings A and D (Fig. 3-5B). The compact structure was derived from IR and NMR observations and confirmed by X-ray crystallography (Bonnett *et al.*, 1978). More recently it has been demonstrated by resonance Raman spectra (Yang *et al.*, 1991). Owing to this isomerization the solubility of bilirubin in aqueous solution is only about 7 n*M* at pH 7.4, 37°, far lower than would be expected for a hydrophilic compound, but still about 100-fold above that of LCFAs.

The affiliation with albumin was detected in the 1930s by showing that bilirubin migrated with albumin on electrophoresis and ultracentrifugation, and it was confirmed by demonstration of bilirubin with crystalline albumin from the laboratory of E. J. Cohn in 1949 (Martin, 1949). The characteristic red shift in the bilirubin UV spectrum on binding was noted at the same time. Obviously the association is tight enough to survive rigorous treatment. Binding of bilirubin and its immediate oxidation product, biliverdin, is a characteristic of serum albumins throughout evolution even back to the bony fishes (Fang and Bada, 1990) or lamprey (Fellows and Hird, 1982), but the affinity varies widely among species.

1. Affinity Constants

Affinity constants for bilirubin–albumin association have been measured by many methods, most of them titrimetric with a spectral, fluorescent, ESR, or

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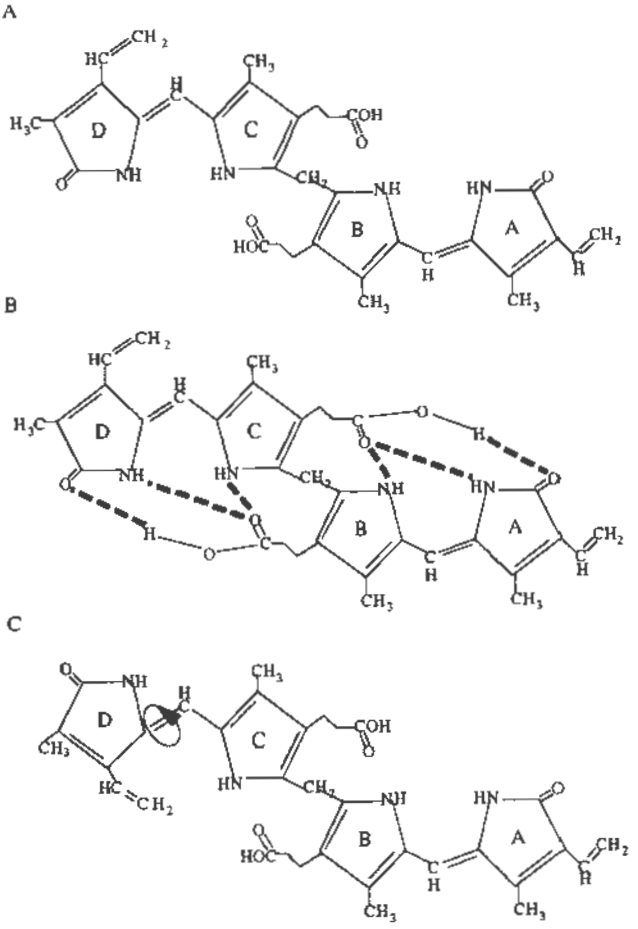


Fig. 3-5. Structure of bilirubin IXα molecule: (A) *ZZ* form in planar presentation; (B) *ZZ*, folded, with hydrogen bonds; (C) *ZZ* $\rightarrow$ *ZE* conversion. Based on the work of Brodersen (1980).

optical rotatory end point. A more direct procedure was developed by Jacobsen whereby the concentration of free bilirubin is determined by the rate of its oxidation by peroxidase; bilirubin bound by albumin is generally protected from such oxidation, but caution is advised to ascertain the validity of this assumption, because hemoglobin and some drugs interfere (Brodersen *et al.*, 1979). Rat

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albumin does not afford such protection, so the method is not applicable with this species (Frandsen and Brodersen, 1986).

Bilirubin is unlike LCFAs in having a single primary site on albumin that is much stronger than subsequent site(s). The "most probable value" from a review of 19 studies by Brodersen (1982) is $K_A = 9.5 \times 10^7\ M^{-1}$ for a single primary site at 37° and pH 7.4 (Table 3-1). Further analysis suggests a secondary site only 1/30 as strong, $K_A = 0.3 \times 10^7\ M^{-1}$. This difference in affinity means that bilirubin at $\bar{\nu} < 1$ will not be distributed among sites as are LCFAs; only 1–2% will occupy secondary sites (Wosilait, 1974). The binding rises with pH and decreases with chloride concentration (Jacobsen, 1977). Adjustment of the pH to 7.4 with CO_2 is thus recommended before measuring bilirubin affinity in plasma (Yeung *et al.*, 1992).

Fortuitously, the affinity of bilirubin for agarose lies between that of the first and second sites on HSA; this allows easy removal of bilirubin exceeding the capacity of the first site by passage over a column of Sephadex, and is applied in determining the "reserve capacity" remaining before the first site is saturated. Only the bilirubin bound at this primary site is prevented from crossing into the central nervous system of neonates and causing kernicterus (Chapter 6, Section II,B,6).

2. *Effects of Binding on Bilirubin Molecule*

When bilirubin binds to albumin its compact structure becomes unfolded and it is held in an extended configuration (Fig. 3-5A) rather than the folded form (Fig. 3-5B). The plane of the AB pyrrole rings is rotated with respect to the plane of the CD pair, causing a marked right-handed chirality (P helicity) with accompanying Cotton effect near 450 nm observed with optical (Blauer and Wagnière, 1975) or fluorescent CD (Thomas *et al.*, 1987). The sign of this change varies among species; it is opposite for HSA and BSA and reverses in HSA at pH 4, suggesting that the shape of the binding pocket is quite adaptable (Blauer *et al.*, 1977). Salt formation with aryl amines will effect a similar chiral change in bilirubin in organic solvents (Lightner *et al.*, 1988a). On the other hand, paired but cleaved dipyrroles such as xanthobilirubin (Jacobsen and Brodersen, 1983) and certain probes (Hsia *et al.*, 1982) will occupy the bilirubin site but do not evoke a chiral change. Biliverdin, which has a conjugated methene system joining the two dipyrrole chromophores, binds at the primary site but less strongly than does bilirubin, and in a left-handed (M helicity) rather than a right-handed twist (Ahlfors, 1981; Trull *et al.*, 1992). On reduction of bound biliverdin with borohydride, the resulting bilirubin converts to the right-handed (P) chirality.

Gossypol, a binaphthyl derivative used as an antibiotic and to promote fertility, has four aromatic rings and binds at the primary bilirubin site with high affinity ($K_A = 1.1 \times 10^7\ M^{-1}$) (Royer and Vander Jagt, 1983). Like bilirubin,

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gossypol shows marked CD Cotton effects on binding, and the sense of the change is different in HSA and BSA (Whaley *et al.*, 1984).

Among other changes noted on binding is a red spectral shift of the bilirubin absorbance peak, the maximum changing from about 440 nm for free bilirubin in chloroform to about 470 nm when bound. The fluorescence of the albumin is quenched whereas that of the bilirubin is enhanced (Beaven *et al.*, 1973). For molar parameters of light absorption, tryptophan fluorescence, bilirubin fluorescence, and CD for bilirubin:HSA ratios of up to 3:1 at pH 8.2, a recent publication of Brodersen's group is recommended (Knudsen *et al.*, 1986). From their absorption spectra they propose that as many as three bilirubins can be internalized.

Bound bilirubin has been found to act as an antioxidant. It protects both the albumin molecule (Neuzil and Stocker, 1993) and the circulation (Chapter 5, Section II,B,3,b) from damage mediated by oxygen radicals.

a. Lumirubin. The well-known sensitivity of bilirubin to light led to illumination of hyperbilirubinemic neonates to expedite bilirubin excretion. Details of the conversion, which occurs on albumin-bound bilirubin, were first studied in 1977 (Pedersen *et al.*, 1977).

In bilirubin IXα *ZZ* the configuration of rings A and B about the double bond at C-4 is the Z ("Zwitterion") form, as is the configuration of rings C and D about the double bond at C-15. (C-4 and C-15 are the carbons of ring A and the last methene bridge, respectively, which participate in the methene bond—Fig. 3-5C.) The conformation is termed the ΔZ4, ΔZ15, or simply the ZZ form. On irradiation at 410–460 nm there is an almost instantaneous (19 psec) conversion around the C-15 bond to the Z4,*E*15 or *ZE* form (*E*, for "Entwicklung") (Greene *et al.*, 1981), with a quantum yield of about 0.2 (Lamola *et al.*, 1982). This isomerization is reversible, but it is followed by a slower structural change to form *EZ*-cyclobilirubin, or "lumirubin"; the isomers are detectable on HPLC (Onishi *et al.*, 1989). Lumirubin is less strongly bound to HSA and, because it cannot form internal hydrogen bonds, is significantly more soluble in plasma and is readily excreted by the kidney.

When studied *in vitro* the optimal wavelength for excitation is near 510 nm; quantum yield was 0.11 at 458 nm at 23° (Agati *et al.*, 1992). The optimal wavelength for use with infants is different owing to light-absorbing effects of the skin. Co-binding of LCFAs was reported to increase the quantum yield through an allosteric effect (Irollo *et al.*, 1987). Another study found that the instantaneous conversion to the *ZE* form proceeds best at the primary bilirubin site, but that the cyclization step is favored at the secondary site (Onishi *et al.*, 1989). This would predict that bilirubin held in excess of the primary site would be more rapidly eliminated.

b. δ-Bilirubin. Bilirubin is normally conjugated with glucuronate in the hepatocyte in preparation for biliary secretion. If the secretion of conjugated

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bilirubin is impaired, as in cholestasis, and conjugation continues, some of the bilirubin can transfer one of its propionate carboxyls from glucuronate to an albumin lysyl ε-amino group (Weiss *et al.*, 1983).

This covalently bound bilirubin was termed δ-bilirubin because it appeared as the fourth peak on an HPLC separation of bilirubin conjugates. It will form slowly *in vitro* from albumin and bilirubin diglucuronide (Gautam *et al.*, 1984; Adachi *et al.*, 1991); *in vivo* it is probably produced within a damaged hepatocyte to which circulatiog albumin has gained access. The δ-bilirubin is held in the normal primary binding site by an amide bond, so this is a form of natural affinity labeling. If the glucuronate is indeed the activating group for the propionate carboxyl, this phenomenon demoostrates that a molecule of the size of glucuronate does not sterically hinder binding of bilirubin at its primary site, implying that the ε-lysyl residue forming the salt bond with unconjugated bilirubin is near the surface or is made accessible through unfolding of the albumin. Clinical aspects of δ-bilirubin are discussed in Chapter 6 (Section II.B.4.a).

3. Nature of Binding Site

The binding reaction is strongly hydrophobic, as is LCFA binding, but with a negative entropy change and larger negative change in enthalpy, $\Delta G = -11.0$, $\Delta H = -13.5$ kcal mol $^{-1}$, $\Delta S = -8.4$ cal mol^{-1} deg (Jacobsen, 1977). These data indicate the importance of hydrogen bonds and salt linkages rather than of hydrophobic forces in the binding mechanism, consistent with the picture of the captured bilirubin molecule as an open structure with its hydrophilic groups available for bonding. Salt linkages are reputedly less important for binding than they are for orientation, because blocking the carboxyl groups as methyl esters causes changes in the CD spectrum but not in the association constant (Lightner *et al.*, 1988b).

Chemical modification studies have variously implicated lysyl, arginyl, histidyl, and tyrosyl groups in bilirubin (Jacobsen *et al.*, 1972). pH-jump tests were interpreted to mean that 1 to 3 fast-ionizing tyrosines became slow ionizing (Honoré and Brodersen, 1992). Participation of lysyl residues that are buried rather than exposed has been suggested by a two-step blocking procedure; this result supports a model in which the bilirubin site is normally buried but becomes accessible through a series of relaxation steps on interaction with the ligand. Indeed, stopped-flow observations of fluorescent spectral changes report an almost instantaneous interaction of a single bilirubin with HSA, then a series of slower first-order changes of $k = 40$–50, 4–8, and ≈ 2 s^{-1}, extending as long as 500 s at 6° (Gray and Stroupe, 1978; Jacobsen and Brodersen, 1983). These delayed changes are not observed with dipyrroles so are apparently related to accommodation of the large bilirubin molecule by the albumin.

The search for the primary bilirubin site was directed to the region of loop 4, residues 198–308, in subdomain IIA, by testing the binding ability of isolated

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fragments of HSA and BSA (Table 2-2). Affinity labeling with bilirubin activated by Woodward's reagent K narrowed the location of participating cations to residues 195–251 of HSA (Reed and MacKay, 1985); Jacobsen (1978) found that bilirubin couples by a carbodiimide to Lys-240 in peptide 240–258. Inhibition of binding of DNS-aziridine at Lys-220 by bilirubin suggested that this residue lies in the site in BSA (Brown and Shockley, 1982); the corresponding cation in HSA is Arg-222, which lines the opening to Site I (Fig. 2-9). Other cationic residues at the site are Lys-199, Arg-257, and His-242.

Several probes have been used to study the properties of the bilirubin sites. Hsia *et al.* (1982) used D and L forms of a glutamate-oxylpiperidine compound to demonstrate stereospecific binding of these two spin labels at three bilirubin sites, and proposed allosteric enhancement by the first site on a second site. Contrary to this interpretation, however, Sato *et al.* (1988) found bilirubin binding to be anticooperative at all steps. A fatty acid derivative, 11-(dansylamino)undecanoic acid, is claimed to bind at MCFA and bilirubin sites rather than LCFA sites (Wilton, 1990). The dipyrrole, xanthobilirubin, was mentioned above. Mono acetyldiaminodiphenyl sulfone (MADDS) is used as a bilirubin surrogate to measure reserve binding, but its specificity has been claimed to be closer to hematin than bilirubin (see Section I,B.4) (Ho *et al.*, 1985).

4. *Hematin and Other Porphyrins*

Because the porphyrins are the direct metabolic precursors of bilirubin and there has been concern that hematin would interfere with albumin binding of bilirubin in neonates, it is appropriate to consider porphyrin binding at this point. The concern proved to be without foundation; hematin at $\bar{\nu} = 0.2$–0.6 did not displace bilirubin from cord blood saturated with bilirubin (Kirk *et al.*, 1984). Despite their similar compositions, the flat ring structures of porphyrins must look very different to a prospective binding site than the extended, twisted conformation of bound bilirubin. Predictably, the primary sites for the two ligands do not cross-over in specificity; each has been shown to be strong and distinctive (Hrkal and Klementová, 1984; Ho *et al.*, 1985). Koskelo and Muller Eberhard (1977) have reviewed the binding of porphyrins.

In primate albumins there is a single primary site for hematin, with $K_A = 1.1 \times 10^8\ M^{-1}$ at pH 7.5 (Table 3-1). Most values for the association constant have been calculated from Scatchard plots of the change in Soret band fluorescence (activation at 405 nm); they range as low as $3.6 \times 10^6\ M^{-1}$ (Reddi *et al.*, 1981). Secondary porphyrin sites to a maximum $\bar{\nu} = 3$ have been reported (Rotenberg *et al.*, 1987); they are much weaker and do show overlap with binding at the secondary bilirubin site(s) (Hrkal and Klementová, 1984). Subprimate albumins other than the frog appear not to have the strong hematin site.

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Kinetically, the uptake of hematin by HSA shows a very rapid association, then a slower molecular rearrangement common to the uptake of other organic compounds (Adams and Berman, 1980). This slower phase is entropy driven: $\Delta G = -10.8$, $\Delta H = 0$ kcal mol^{-1}, $\Delta S = +34.9$ cal mol^{-1} deg^{-1} at 37° (Rotenberg *et al.*, 1987), implying hydrophobic bonding. CD changes suggest π–π^* interactions with aromatic side chains of the albumin. The affinity falls as the pH approaches 8, possibly owing to the N → B isomerization of the albumin molecule (Reddi *et al.*, 1981).

A metal component in the porphyrin has little effect on the binding. The affinity for protoporphyrin without a central metal ion is a little less than that for hematin, $K_A = 1.4 \times 10^6\ M^{-1}$. K_A values for Fe(II), Fe(III), and Zn(II) porphyrins are 3.6, 3.2, and 4.7 $\times$ $10^6\ M^{-1}$, respectively (Reddi *et al.*, 1981). In serum, however, presumptive reduction of metheme–albumin with dithionite causes loss of the hematin to hemopexin, suggesting that the ferrous form is less weakly bound (Morgan *et al.*, 1976).

Substitution for the two hydrophilic -CHOHCH$_3$ side chains of either -CH=CH$_2$ or -H alone raises the K_A markedly to 280 $\times$ 10^6 or 450 $\times$ $10^6\ M^{-1}$, respectively (Rotenberg *et al.*, 1987), and implies even greater importance of hydrophobic binding. Hematoporphyrin ester binds with $K_A = 3.6 \times 10^6$; its dimeric and trimeric forms, potentially useful for tumor photosensitization, bind slightly less strongly (Rosenberger and Margalit, 1993).

The most avid carrier of hematin in plasma is the specific heme-binding protein, hemopexin. Uptake of hematin to hemopexin is 30 times as rapid as to albumin, and hematin will readily leave albumin for hemopexin in plasma. It must be remembered, however, that the concentration of hemopexin is only about 1/400 of that of albumin; the initial binding is to hemopexin and albumin acts as a depot for the overflow. Hemopexin has a specific receptor mechanism for delivery of hematin to the liver that is distinct from that of albumin (Naitoh *et al.*, 1988). Protoporphyrin, however, is reported to be carried 90% on albumin and only 1% on hemopexin, so the iron ion would appear to be more important in binding to hemopexin than to albumin (Lamola *et al.*, 1981).

Direct evidence is lacking for a location of the primary hematin site on the albumin molecule. Neither bilirubin, LCFA (Beaven *et al.*, 1974), nor diazepam disturbs the binding of 1 M/M of hematin; confusingly, the reputed bilirubin probe, MMADS, did interfere, raising some doubt about its validity as a bilirubin analog (Ho *et al.*, 1985). The finding of Hrkal *et al.* (1978) suggest that the middle CNBr-produced HSA fragment, 124–298, loops 3–6, binds hematin most strongly, although still less so than does whole albumin. Fluorescence energy coupling data imply that the hematin site lies within 17 Å of the Trp-214 in loop 4 of HSA, so that the region of loops 3–4 seems a presumptive location of the primary, specific hematin site.

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C. Site-I Ligands

So many different compounds are believed to bind in the region termed Site I by Sudlow *et al.* (1976) that they will be considered here together despite their great diversity. Many of them are therapeutic drugs, the binding of which there is a practical reason to study. Until the tertiary structure of HSA was known from X-ray studies, hard data on the location of Site I were lacking. Sharing of the locus by different ligands was known only through studies of competition. Now it appears that Sudlow's Site I is indeed located in domain IIA, as had been suspected from physical studies, embracing residues from Lys-199 through Glu-292 (Table 2-5)— more on this later.

Ligands frequenting this region fall broadly (and rather loosely) into several groups: bilirubin, warfarin, salicylates, and a large miscellaneous category. The latter includes the previously considered cyclic eicosanoids and hematin as well as ω-dicarboxylic medium-chain fatty acids (Tonsgard and Meredith, 1991), although the monocarboxylic MCFAs bind in Sudlow's Site II. Typically Site I ligands are bulky heterocyclic anions with the charge situated in a fairly central position in the molecule. This differentiates them from the ligands typical of Site II, located in domain IIIA and considered in the following section, which are generally aromatic and may be neutral; a charge, if present, is anionic and located more peripherally on the molecule.

From the diversity of acceptable ligands and the apparent ability to accommodate more than one of them at a time without significant interference, leading students of drug binding, such as Kragh-Hansen (1988) and Fehske *et al.* (1981), have termed this site "a large and flexible region" or a complex of "different but overlapping sites" (Fehske *et al.*, 1982). Site I is the one primarily affected by the basic **N** → **B** transition near pH 8; even in serum this conformational change in the albumin molecule causes tighter binding of warfarin, bilirubin, and several drugs (see Chapter 2, Section II,C,1,c).

Table 3-5 lists association constants for a mere sampling of the myriad drugs and other nonphysiological compounds that albumin binds in Site I and Site II. Most of these exhibit K_A values of only 10^3 to $10^5\,M^{-1}$, weaker than the LCFAs and bilirubin (Table 3-1) but sufficient to limit the free form in plasma to 1–10% of the total concentration, so that the albumin has an important effect in buffering changes in the concentration of the free drug and limiting its rate of metabolism and excretion. With hypoalbuminemia, for example, the rate of adverse reactions to warfarin, prednisone, and phenytoin is markedly elevated. Displacement of therapeutic agents by other drugs, such as antiinflammatory agents, analgesics, and radiocontrast media (Doweiko and Nompleggi, 1991b), is another concern. Note the high affinity for iophenoxate (Table 3-5), a binding so high that this contrast agent was abandoned because it is eliminated so slowly from the body (Mudge *et al.*, 1978).

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TABLE 3-5
Binding of Drugs and Other Exogenous Compounds

Compound	Association constant, K (M^{-1})	n	Percent free[a]	Reference
Site I				
Salicylate	1.9×10^5	1	16	Kragh-Hansen (1988)
Sulfisoxazole	1.8×10^5	1	15	Anton (1973)
Warfarin, *S*-(−)	3.3×10^5	1	3	Pinkerton and Koeplinger (1990)
Phenylbutazone	7.0×10^5	1	1	Kragh-Hansen (1988)
Digitoxin	0.4×10^5	1	3	Kragh-Hansen (1981)
Indomethacin	1.4×10^6	1	1	Montero *et al.* (1986)
Tolbutamide	4×10^4	1.4	1	Vallner (1977)
Furosemide	2.6×10^4	1.6	3	Viani *et al.* (1991b)
Phenytoin	6×10^3	6	9	Schoenemann *et al.* (1973)
Chlorpropamide	3.3×10^5	1	4	Kragh-Hansen (1988)
Chlorthiazide	3.1×10^4	2	11	Kragh-Hansen (1981)
Oxacillin	4.7×10^3	1	6	Joos and Hall (1968)
Benzylpenicillin	1.2×10^3	1	75	Joos and Hall (1968)
Acetotrizoate	4×10^4	1	--	Pinckard *et al.* (1973)
Phenol red	2.8×10^4	1	—	Rodkey (1961)
Bromcresol green	7×10^5	3	--	Rodkey (1964)
Bromphenol blue	1.5×10^6	3	—	Bjerrum (1968)
Iophenoxate	8×10^7	—	—	Mudge *et al.* (1978)
Sulfobromophthalein	1.7×10^7			Baker and Bradley (1966)
Methyl orange	2.2×10^3			Klotz *et al.* (1946)
Methyl red	2.2×10^5	–	—	Burkhard *et al* (1961)
Evans blue	4.0×10^8	14	—	Freedman and Johnson (1969)
Site II[b]				
Diazepam (S)	3.8×10^5	1	1	Kragh-Hansen (1991)
Ibuprofen	2.7×10^6	1	—	Kragh-Hansen (1981)
Naproxen	1.2×10^6	1	<1	Honoré and Brodersen (1984)
Octanoate	5.5×10^5	1	—	Honoré and Brodersen (1988)
Clofibrate	7.6×10^5	1	10	Meisner and Neet (1978)
Cationic drugs				
Chlorpromazine	2.0×10^5	—	-	Kragh-Hansen (1981)
Imipramine	2.5×10^4	—	—	Kragh-Hansen (1981)
Quinidine	1.6×10^3	—	---	Kragh-Hansen (1981)

[a]Some data from Wandell (1983).
[b]See also tryptophan and thyroxine (Section III.A.4) as Site-II ligands.

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It is not feasible to provide a comprehensive tabulation of the affinities of albumin for therapeutic drugs in this volume. For drugs not in Table 3-5 the reader is referred to the vast pharmacological literature, and to the reviews by Vallner (1977) and Kragh-Hansen (1981). Some general reviews of albumin/drug binding are those of Jusko and Gretch (1976), Koch-Weser and Sellers (1976), Fehske *et al.* (1981), Lindup (1987), Kragh-Hansen (1990), and an impressive work by Goldstein (1949), which is still useful after nearly 50 years.

Many literature reports have depicted the relationships among different Site-I ligands. Colchicine does not bind, but deacetylcolchicine does ($K_A = 8 \times 10^3$ M^{-1}) and is blocked by salicylate (Trnavska *et al.*, 1979). Phenol red competes with bilirubin for binding, but not with warfarin or digitoxin (Kragh-Hansen, 1985). The displacement of bilirubin by some sulfa drugs has long been known (Goldstein, 1949). Interference between warfarin, phenylbutazone, and the classical marker for Site I, dansylamide, puts these substances in the same binding region within the site; digitoxin is distinct from both of these groups, but conflicts with salicylates (Kragh-Hansen, 1985). Hence, three primary regions or subsites can be described in the Site-I complex: bilirubin–phenol red, warfarin–phenylbutazone, and digitoxin–salicylate (Kragh-Hansen, 1990).

Interference studies point out broad areas of overlap (Fehske *et al.*, 1982). Indomethacin, which would seem to belong with other indoles in Site II but instead binds at Site I, phenytoin, furosemide, and tolbutamide all contend with both phenylpropazone and salicylates (Lindup, 1987; Viani *et al.*, 1991a), and indomethacin also competes with bilirubin (Rasmussen *et al.*, 1978). Aspirin is known to displace bound bilirubin. Although it is difficult to compare many of the reports owing to differences in methodology and experimental conditions, and some of the results may be attributable to secondary conformational effects rather than to direct competition, we cannot escape the implication that this amidships cargo hold in the albumin molecule is large and flexible, a multiple locus, willing to adapt to nearly all comers and even to take in more than one if space permits. It is Site I that is largely responsible for the cosmopolitan reputation of albumin among transport proteins.

1. Dyes and Other Reporter Compounds

Dyes are popular ligands with which to assess the properties of albumin because they are readily perceptible and often undergo visible color changes on binding—the so-called protein error that confused protein chemists attempting to measure pH. As long ago as 1921 the association of injected dyes such as Congo red with plasma proteins was recognized (Bennhold, 1961), and Goldstein in 1949 listed 54 dyes that had been observed to bind to albumin. I.M. Klotz of the E.J. Cohn group used methyl orange and methyl red in studying the physical chemistry of ligand binding as measured by dialysis (Klotz *et al.*,

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1946, 1952). Karush (1952), again using methyl red, first noted differences in binding affinities of optically isomeric forms. Phenol red and bromcresol green (BCG), the latter to become a favorite analytical reagent for albumin, were studied by Rodkey (1961, 1964). Bromcresol purple (BCP) is another popular analytical reagent (Louderback *et al.*, 1958). 2-(4'-Hydroxyphenylazo)benzoic acid (HABA) (Ness *et al.*, 1965), used similarly until interference by bilirubin was discovered, and 10 other azobenzene derivatives were studied by Baxter (1964). Bromphenol blue binds at three sites (Table 3-5), the first of which coincides with the primary bilirubin site (Bjerrum, 1968).

Among the most tightly bound dyes is sulfobromophthalein (BSP) (K_A = $1.7 \times 10^7\ M^{-1}$, Table 3-5); its elimination after intravenous injection was used for decades as a measure of hepatic function until its toxicity became apparent. Evans Blue was similarly used as a measure of plasma volume, binding to albumin restricting its loss into extravascular spaces. Cibacron Blue, discussed below, is helpful in the isolation of albumin. Dansylamide and dansylglutamine have been employed as markers of Site I (Kasai *et al.*, 1987), and He and Carter (1992) used triiodobenzoate as a heavy-atom ligand for both Site I and Site II.

2. *Specificity of Site I*

It is difficult to describe a typical Site-I ligand, considering their diversity. Those listed in Table 3-5 are, as noted earlier, generally heterocyclic anions, not aromatic except for a few phenyl groups. Some of the dyes are exceptions; these are usually heavily halogenated compounds, and actually their assignment to Site I is not firm. A region that will confine bilirubin, warfarin, salicylate, phenylbutazone, indomethacin, various penicillins, furosemide, and radioopaque dyes such as iophenoxate must be a marvel of adaptability.

Selectivity is seen in some respects, however. *m*-Methyl red is held differently than are the *ortho* and *para* forms (Burkhard *et al.*, 1961). Chiral specificity is apparent. Both stereoisomers of warfarin are bound by HSA, but the *R*-(+) is more readily displaced by meclofemate whereas the *S*-(−) form is more readily displaced by phenylbutazone (Li *et al.*, 1988). K_A for the *R*-(+) form is $4.4 \times 10^5\ M^{-1}$, 33% higher than that for the *S*-(−) form (Table 3-5).

Among animal species there are widespread differences in relative affinities for organic compounds. The inaccuracies inherent in using BCG, BCP, or HABA to assay albumin in different animals are well known, as are the poor yields obtained on isolating rat or chicken albumins with Cibacron Blue (Naval *et al.*, 1982). Cibacron Blue apparently binds at the bilirubin site in HSA but not in BSA (Leatherbarrow and Dean, 1980). Dog, cow, horse, and sheep albumins bind warfarin and dansylsarcosine similarly to HSA, but rat albumin does not (Panjehshahin *et al.*, 1992). Brodersen's group found that dog, pig, rabbit, hamster, rat, and cat albumins all differed from HSA in affinity for MADDS (the purported bilirubin model compound) and sulfonamides (Robertson *et al.*,

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1990). The implication from these findings is that relatively small substitutions in amino acid sequence (Chapter 4, Section III) can be important in determining the observed specificities in Site I.

3. Effects of Binding on Albumin Molecule

Conformational changes in the host albumin molecule are evident with many Site-I ligands. The CD spectra exhibit Cotton effects with sulfonamides (Müller and Wollert, 1976), warfarin (Brown and Müller, 1978), indomethacin (Perrin and Nelson, 1972), and phenylbutazone (Watanabe and Saito, 1992). Changes in UV absorption are generally apparent in difference spectra. Methyl orange increased the stability of HSA to attack by five proteases, trypsin, chymotrypsin, papain, subtilisin, and pronase (Markus *et al.*, 1967a); this result was taken to mean that the "configurational adaptability" involves more than the immediate vicinity of the ligand and can affect the compactness of structure of the whole albumin molecule.

Oida (1986b) found ^{1}H NMR spectral effects indicative of configurational alterations of HSA for many of the Site-I compounds of Table 3-5, including warfarin, phenylbutazone, tolbutamide, chlorpropamide, salicylates, digitoxin, clofibrate, phenytoin, indomethacin, BCG, and dansylamide. Nonspecifically bound drugs such as haloperidol did not produce conformational changes.

Kinetic measurements by the stopped-flow technique showed, as with several previously discussed ligands, that the initial step in warfarin binding is extremely rapid, diffusion controlled, and that the second step is a slower adaptation of the protein site. The second step is faster in binding to a fragment (1–387) than to whole albumin, suggesting some clumsiness of adjustment in the larger albumin structure (Bos *et al.*, 1989a). Similar results have been seen with binding of several dyes (phenol red, methyl orange) and ANS (Nakatani *et al.*, 1974).

Thermodynamic parameters support the concept, as with other organic ligands, that the conformational adaptations are largely entropy driven (Aki and Yamamoto, 1989). ΔH and ΔG are usually relatively small or negative, and ΔS positive. Thus, for warfarin and oxyphenylbutazone at 37°, values were, respectively, ΔH, −4.0 and 2.5; ΔG, 7.2 and 7.2 kcal mol^{-1}; and calculated ΔS, +10.3 and +15.2 cal mol^{-1} deg^{-1} (Dröge *et al.*, 1985b).

On binding of aspirin in Site I an esterase action occurs and the acetyl group is transferred to nearby Lys-199. First noted by Hawkins *et al.* (1969), the lysine site was identified by Walker (1976a) shortly after the HSA sequence was known, by isolation of the peptide Leu-Lys*-Cys-Ala-Ser-Leu-Gln-Lys following tryptic digestion. About 85% of [^{14}C]acetylsalicylate was found in the Lys-199 (denoted by an asterisk). Lawson *et al.* (1982) found two antithrombin drugs to be inactivated by covalent binding to Lys-199 as well.

The esterase, or rather transacetylase, action has been documented by ^{1}H NMR (Honma *et al.*, 1991), and the appearance of the acetyl group of aspirin as

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an acetamide confirmed by ^{13}C NMR (Gerig *et al.*, 1981). Lys-199 was found to be well exposed to the solvent, and its ϵ-NH_2 to have an abnormally low pK of ≈8, perhaps the result of the proximity of His-242, just across an S–S bond (He and Carter, 1992). The methyl group of the attached acetamide shows essentially unrestricted rotation, $\tau_1 < 0.1$ nsec. Salicylate anion blocks the acetylation, showing that the transfer reaction requires restraint of the aspirin in the specific site; predictably, indomethacin also blocks the reaction through its competition for the binding site. Although many proteins have been reported to be acetylated by aspirin, circulating albumin is the predominant target *in vivo*.

Trinitrobenzenesulfonate and related compounds bind in Site I as well; in this case the trinitrophenyl group is transferred to a lysyl ϵ-NH_2 group. Of two highly active lysines, one has been identified as Lys-199 through effects on the fluorescence of the nearby Trp-214 (Kurono *et al.*, 1983a) and through ^{19}F NMR experiments (Gerig *et al.*, 1978). Again there is an esterase activity on the reversibly bound ligand; cleavage of the Meisenheimer complex, which forms on reaction with trinitrobenzenesulfonate and sulfite, was observed by Taylor and Vatz (1973), and traced to Lys-220 of BSA. (Lys-199 of HSA is replaced by Arg-198 in BSA, and a basic residue homologous to BSA Lys-220 is HSA Arg-222.) Both Taylor's (Sturgill *et al.*, 1977) and K. Ikeda's groups (Kurono *et al.*, 1992) have made serial studies of the esterase activity of HSA and BSA. Characteristically it is slow, with $k_c = \sim 0.3 s^{-1}$, well below that of a true enzyme.

Several penicillins also link covalently to HSA, a phenomenon discovered as the cause of a transient bisalbuminemia appearing clinically in patients receiving either penicillin G (Arvan *et al.*, 1968) or benzylpenicillin (Lapresle and Wal, 1979). The effect could be duplicated by incubation of serum *in vitro*. Characteristically the active drugs were "penems" (Fig. 3-6), β-lactams with key features of both cephalosporins and penicillins but without an amido substitution in position 6 (Bruderlein *et al.*, 1981). Irreversible binding to albumin is proposed to occur through acylation of an ϵ-lysine amino group with concurrent rupture of the β-lactam ring between the carbonyl carbon and the nitrogen atom. Oddly, it appears that prior reversible binding of the lactam to the albumin is not required for the reaction *in vitro* (Bundgaard, 1977), although some of the coupling can be blocked by dansylamide, a Site-I marker.

The primary site of penicilloyl coupling appears to be the familiar Lys-199 of HSA, as with several Site-I ligands. Penicilloyl groups have also been detected on nearby Lys-190 and Lys-195, and on Lys-432, -541, and -545 in domain III of HSA (Yvon *et al.*, 1990). Fixation at His-146 and -338 was suggested by another study through somewhat indirect evidence (Lafaye and Lapresle, 1988b). Hence this covalent coupling may be specific only for surface-available nucleophilic groups, particularly lysines.

Clinically the coupling can be significant. Approaching 100% labeling of albumin molecules, the "bisalbuminemia" is associated with the appearance of antipenicilloyl antibodies. These antigenic sites, the major epitopes for penicillin

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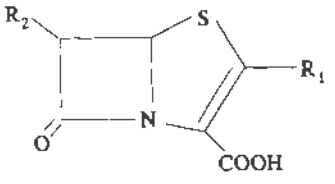


Fig. 3-6. Structure of penem group.

allergenesis (see Chapter 6, Section II,B,5,b), are unusual in that they were described as sheltered, demonstrable only after proteolytic digestion (Lafaye and Lapresle, 1988a).

4. Location of Site I

The X-ray findings of He and Carter (1992) showed the binding cavity corresponding to Site I to lie in subdomain IIA (Fig. 3-1). The residues involved, as defined by interactions with triiodobenzoate, which probably does not include all of the interactions with the variety of ligands bound in this site, are listed in Table 2-5 and are indicated in Fig. 2-9. They lie chiefly in helices h2, h3, h4, in the long loop of subdomain IIA, and h6 of its short loop; Glu-292 is at the edge of h6. Details of orientation of aromatic rings vary with ligand, e.g., salicylate or triiodobenzoate; the COO^- group of triiodobenzoate orients between Lys-199, Arg-257, and His-242. Carter and Ho (1994) describe the van der Waals surface of the binding site as "an elongated sock-shaped pocket wherein the foot region is primarily hydrophobic and the leg is primarily hydrophilic" and accessible to the solvent.

Supporting biochemical evidence of the location of Site I includes the above-described propensity of Lys-199 for attachment of Site-1 ligands. The importance of Trp-214 was attested by the blocking of binding of warfarin on modification of this tryptophan (Fehske *et al.*, 1982). A large peptic fragment of HSA, 1–387, bound warfarin with practically the same affinity constant as did whole albumin (Bos *et al.*, 1988c); the authors concluded that the primary warfarin site is in domain II and that domain I plays a part, because the affinity for tryptic peptide 198–585 was lower than for whole albumin.

Other evidence concerns the effects of substitutions at known amino acid residues—nature's mutagenesis experiments (see Chapter 4, Section IV,D, and Fig. 4-8.) The findings are not as helpful as might be hoped. One study concluded that warfarin and salicylate are both bound in domain II (Vestberg *et al.*, 1992). In another, the 269 Asp → Gly mutation was observed to depress salicylate binding (Kragh-Hansen *et al.*, 1990a). A general depression of salicylate, warfarin, and benzodiazepine binding by mutations in the 313–365 region was

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attributed to conformational changes. An 186 Arg → Glu substitution in the macaque (Watkins *et al.*, 1993) depressed bilirubin binding, again possibly the result of a conformational change.

D. Site-II Ligands

Sudlow's studies of competitive binding established Site II as a discrete locus for certain drugs, with dansylsarcosine as a marker, but did not assign it to a region of the albumin molecule. Diazepam, flufenamate, iopanoate, ethacrynate, naproxen, and chlorophenoxyisobutyrate (clofibrate) are now among the Site-II drugs (Sollenne and Means, 1979).

In 1963 Sanger identified the sequence Arg-Tyr*-Thr-Arg as the site of specific labeling of BSA by diisopropyl fluorophosphate, the classical inhibitor of serine proteases. When the albumin sequences became known, the tyrosine was recognized as Tyr-410 of BSA or Tyr-411 of HSA, near the tip of long loop 7, a site that Means and Wu (1979) showed is the residue acetylated in the course of esterase activity albumins toward *p*-nitrophenyl acetate. Stoichiometric inhibition of this esterase activity by several of the Site-II drugs and by L-tryptophan, diazepam, and C_6–C_{10} fatty acids localized other ligands to this area (Koh and Means, 1979; Ikeda *et al.*, 1979). Morávek *et al.* (1979) found Tyr-411 to be the tyrosine most susceptible to nitration, and the nitration to inhibit tryptophan and diazepam binding (Fehske *et al.*, 1979).

The nearby Lys-413 of BSA (Lys-414 of HSA) became implicated in the site by its facile reaction with trinitrobenzene sulfonate or *N*-dansylaziridine (Brown and Shockley, 1982). Dansylation at this lysine blocks tryptophan binding (Jacobsen and Jacobsen, 1979). L-Thyroxine appears to share the tryptophan site on the basis of competitive studies (Tritsch and Tritsch, 1963) and to be similarly dependent on the positive charge of Lys-414 in HSA.

Even before the albumin sequence was disclosed, King and Spencer (1970) had isolated the large, C-terminal cyanogen bromide fragment of BSA that retained near full binding activity for L-tryptophan and octanoate (Table 2-2). Later, binding activity of the large tryptic HSA fragment, 198–585, allowed the prediction that the diazepam site is in domain III (Sjödin *et al.*, 1977b; Bos *et al.*, 1988a).

The effects of single-residue mutations (see Fig. 4-8) have again not been very helpful in pinpointing the binding site. Binding of both warfarin and diazepam is diminished with alterations at residues 313, 321, 365, 570, and 580 (Kragh-Hansen *et al.*, 1990a; Vestberg *et al.*, 1992); thyroxine binding was unaffected by substitutions at 269, 313, 321, 365, or 570 (Kragh-Hansen *et al.*, 1990b). Most of the effects are probably nonspecific and related to tertiary structure modifications.

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We see that a modest body of evidence places Site II in subdomain IIIA, where it has been more precisely defined by X-ray crystallography. Its ligands are L-tryptophan, L-thyroxine, octanoate, diazepam and other benzodiazepines, iopanate, clofibrate, and nonsteroidal antiinflammatory drugs such as ibuprofen and naproxen. Affinity constants for some Site-II ligands are given in Tables 3-1 and 3-5.

1. Tryptophan and Other Indoles

Tryptophan, the largest amino acid, is the only one that is significantly bound by serum albumin, if we regard thyroxine as primary a hormone rather than an amino acid. Much of our information on its binding comes from the 1958 doctoral thesis of R.H. McMenamy at the Harvard Physical Chemistry Laboratory and subsequent publications at the University of Buffalo. Its binding is loose, $K_A \approx 1 \times 10^4\ M^{-1}$ at 37° (Table 3-1), so that only about 75% of circulating tryptophan is bound.

The affinity of albumin for *L*-tryptophan, as for many hydrophobic ligands, rises with decreasing temperature; at 20° K_A is $4.4 \times 10^4\ M^{-1}$ (Kragh-Hansen, 1991). Optimal pH of binding at 37° is 8.7. Chloride ion competes through a weak binding (described below). The association is strongly chiral, D-tryptophan binding only 1% as strong (McMenamy and Oncley, 1958). This property enables separation of the tryptophan enantiomers on HSA immobilized on a solid support; for these separations the optimal pH is 7.8 and the optimal temperature is 24° (Gilpin *et al.*, 1991).

Modifications to the tryptophan molecule alter the affinity. Substitution of a methyl group for the α-hydrogen blocks binding, and decarboxylation to tryptamine reduces it over 20-fold. The latter effect correlates with the poor binding of cationic ligands at Site I and Site II, seen also with poorer binding of skatole or of methyl or ethyl esters of tryptophan. *N*-Acetyl-L-tryptophan, not unexpectedly, binds about 40% more tightly than the zwitterionic form (McMenamy and Oncley, 1958), and indole propionate nearly 25 times more (McMenamy and Seder, 1963). Kynurenine, the opened-ring metabolite of tryptophan, binds surprisingly strongly, $K_A = 2.5 \times 10^5\ M^{-1}$ for BSA at 25° (Churchich, 1972). Transport of tryptophan is apparently restricted to albumins of birds and mammals and was not found in lower species such as fish and lampreys (Fellows and Hird, 1982).

The binding of tryptophan is easily followed by its fluorescence or even its UV absorbance. NMR has shown the 5-fluoro derivative to bind in two chemically distinct sites (Gerig and Klinker, 1980). Selective relaxation rates by ^{1}H NMR reflect that L-tryptophan is less perturbed in its binding site than is its D antipode. The interpretation of this somewhat surprising finding, considering that the L form is held much more strongly, was that the L enantiomer "fits" better into the pocket and so is less constrained (Uccello-Barretta *et al.*, 1991). The

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closeness of the fit of L-tryptophan is indicated by nonbinding of 5-methyltryptophan but good binding of the 6-methyl derivative (McMenamy and Oncley, 1958). Only the phenyl ring and not the pyrrol ring of the indole appears to be involved. Thermodynamic parameters of $\Delta G = -7$, $\Delta H = -2$ kcal mol^{-1}, and $\Delta S = 15$ cal mol^{-1} deg^{-1} predict a strong hydrophobic component (McMenamy and Seder, 1963).

2. Thyroxine

Albumin is for thyroxine only a tertiary carrier, thyroxine-binding globulin and transthyretin both having higher affinities and higher specificities. The loading of albumin is very low, because the total thyroxine concentration in plasma is about 100 n*M* compared to the albumin concentration of 600 μ*M*. [For reviews of thyroxine binding see Cody (1980) and Borst *et al.* (1983).]

It must be noted that a decision whether thyroxine binds at Site I or Site II is still pending. Favoring Site I is competition for thyroxine binding by salicylate, warfarin (Divino and Schussler, 1990), and bilirubin (Kamikubo *et al.*, 1990), all Site-I ligands. The SH group of HSA affects thyroxine binding, the affinity decreasing if the SH (presumably of Cys-34) is blocked as a mixed disulfide with Cys/2 (Ohkubo, 1971). Data suggesting Site II are the reported L-thyroxine/L-tryptophan and L-thyroxine/octanoate competitions for a binding site (Tritsch and Tritsch, 1963; Dalgaard *et al.*, 1989) and the calculated distance from the thyroxine site to the lone tryptophan of HSA, Trp-214, on the basis of fluorescent energy transfer as 22 Å (Perlman *et al.*, 1968); from the tertiary structure Carter (1994) has calculated ≈19 Å, whereas the distance from Trp-214 to the binding pocket of Site I is only ≈12 Å. But we will proceed to consider the properties of thyroxine binding and leave to the future the location of the site.

The K_A of 1.6×10^6 M^{-1} for L-thyroxine (T4) (Table 3-1) is appreciably stronger than that for L-tryptophan. 3,5,3'-L-Triiodothyronine (T3) binds about one-sixth as strongly. The D forms are much less tightly bound, and immobilized BSA is used to effect chiral separations (Chapter 7, Section III,B,5). Structural requirements for competition with thyroxine include an anionic group and a phenyl ring with attached carboxylate (benzoate) plus a highly polarizable substituent, or a phenol plus two such substituents; surprisingly, the binding requires only a single phenyl ring, because triiodobenzoate was bound 22% *more* strongly than thyroxine (Tabachnick *et al.*, 1970). L-Thyronine, with no halogen substituents, appears to bind at a different site; chiral separations of DL-thyronine on immobilized BSA are disrupted by bilirubin, whereas those of thyroxine are disrupted by octanoate (Dalgaard *et al.*, 1989), implicating Site-I and Site-II locations, respectivvely, but at odds with the bilirubin effect on thyroxine binding cited above. The affinity for BSA is essentially the same as that for HSA, and both proteins are used on solid supports in free thyroxine assays.

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The binding of T4 involves a twist in its conformation, with a 120° valency angle between the aryl rings at its ether oxygen atom (Tabachnick *et al.*, 1970). There is a bathychromic shift in the T4 absorbance from its peak at 310 nm (Tritsch, 1968), and CD displays an induced Cotton effect at 319 nm (Okabe *et al.*, 1975). The T4 metabolite, reverse-T3 or 3,3',5'-L-triiodothyronine, binds about one-third as strongly and in a different configuration (Okabe *et al.*, 1989).

The clinical condition of familial dysalbuminemic hyperthyroxinemia, or FDH, is defined by abnormally high (≈double) total T4 levels in clinically euthyroid subjects with normal free T4 (Hennemann *et al.*, 1979). Its cause is a variant albumin allele arising from a single-point mutation, 218 Arg → His (Chapter 4, Section IV.D) not usually detectable by electrophoresis, which has created or strengthened a thyroxine-binding site in subdomain IIA and increased the overall affinity for T4 by about 80-fold (Barlow *et al.*, 1986). The result is that about 30% rather than <6% of T4 is carried by albumin (George *et al.*, 1988). Salicylate and warfarin inhibit T4 binding to the serum in FDH at lower concentrations than in normal serum, and quenching of Trp-214 fluorescence by T4 is increased (Dughi *et al.*, 1993), both effects consistent with the new site being in subdomain IIA. But whether the normal locus of thyroxine binding is Site I or Site II is still not resolved.

3. Octanoic Acid

The medium-chain fatty acids, C_6–C_{14}, do not occur in a significant quantity in the body and so the binding affinity for octanoate is listed in Table 3-5 rather than Table 3-1. Affinity rises by about fourfold per added methylene group between hexanoate and decanoate, but drops off above the C_{10} acid (Koh and Means, 1979).

The primary binding site for these MCFAs is apparently Site II. Binding of the first molecule does not interfere with the binding of the first molecule of LCFA, nor do 1–2 molecules of LCFA affect the binding of MCFAs or the esterase activity of Tyr-411 (Ashbrook *et al.*, 1972; Meisner and Neet, 1978). Each class can share the other's site for secondary binding at higher $\bar{\nu}$, however.

Evidence for octanoate binding in Site II includes the King and Spencer (1970) findings of its interaction with a C-terminal fragment of BSA (Table 2-2). Other testimony is the similar protection provided by octanoate during pasteurization to that afforded by L-tryptophan, competition for binding with many Site-II, but not Site-I, drugs (Wanwimolruk *et al.*, 1983) by MCFA, and interference with the esterase activity of Tyr-411 of HSA (Koh and Means, 1979). The protection against denaturation includes resistance to peptic digestion (Klapper and Cann, 1964) as well as to heat, implying a tightening of the structure of the C-terminal region. As with some other Site-II ligands there is a competition by chloride ion, K_A in 130 m*M* chloride being about one-third that in phosphate buffer (Honoré and Brodersen, 1988).

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A slow cleavage of *S*-lauryl methyl ester has been reported to be highly specific for albumin, and this reaction was even proposed as an assay for HSA by measuring the released 2-mercaptoethanol (Kurooka and Yoshimura, 1973). The reaction involves formation of a thiolactone followed by transfer of the lauryl group to an ϵ-amino group on the protein.

4. Chloride Ion

Binding of chloride ions of albumin has long been recognized (Longsworth and Jacobsen, 1949) (see Chapter 2, Section II.B.2). K_A for chloride is low, 720 M^{-1} (Table 3-1), but the concentration in plasma is high, 100 mM. Careful titrations imply the binding of 7–8 Cl^-/albumin molecule at pH 7.4 (Fogh-Anderson *et al.*, 1993). Affinity for iodide and thiocyanate ions is higher, 6150 and 3500 M^{-1}, respectively (for BSA) (Scathchard and Yap, 1964). For some other anions the order of affinity is chlorate > nitrate > bromide > chloride.

Competition from chlorides is seen mainly with Site-II ligands such as tryptophan. If the implication from ^{35}Cl NMR (Halle and Lindman, 1978) is correct, the chloride ions bind to paired basic residues such as Lys-Lys or Arg-Arg. Of nine such pairs in the HSA sequence, five occur in domain III (beginning at residues 413, 444, 484, 524, and 573); all are in helical regions. Hence much of the chloride binding seems to involve the C-terminal region of the molecule.

Brodersen *et al.* (1990) concluded from the rate of LCFA exchange through a dialysis membrane with whole plasma that there are six LCFA sites of about equal affinity on human albumin rather than six of decreasing affinity. They attributed the difference partly to an effect of chloride ions depressing the strong sites, an effect also noted by Spector (1975), and to enhancement of the weak sites by fatty acids bound to the stronger sites. In the study of Richieri *et al.* (1993), however, no such cooperativity was noted even though isotonic sodium chloride was present, which argues against this effect being important in plasma.

5. Drugs Binding to Site II

The most clear-cut binding of a drug to Site II is that of 2,3-benzodiazepines such as diazepam; the laboratories in Aarhus (Kragh-Hansen, 1990), Stockholm (Sjödin *et al.*, 1976), and Utrecht (Janssen *et al.*, 1985) have been especially active in the study of diazepam binding. Interference by diazepam with binding of tryptophan is definite and easily demonstrated. Kragh-Hansen (1991) recently confirmed the high-affinity binding of octanoate to the indole–diazepam site by equilibrium dialysis.

As with tryptophan, the affinity constant for diazepam of 3.8×10^5 M^{-1} (Table 3-5) declines in the presence of chloride ion, and also of calcium at physiological levels (Wilting *et al.*, 1980), effects that need to be remembered when measuring free diazepam in plasma by dialysis.

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Microcalorimetry yields a ΔH of -5.7 kcal mol^{-1}, with a strong positive effect of pH as in the **N** → **B** isomer transition (Janssen *et al.*, 1985) (see also Chapter 2, Section II,C,1,c). Diazepam binding induces a Cotton effect in CD near 320 nm. Benzodiazepines with a center of asymmetry at C-5 have both central and helical chiralities, and stereoselective binding at Site II is responsible for many of the differences in affinity for various forms of this drug (Visy and Simonyi, 1989; Kaliszan *et al.*, 1992). Benzodiazepines with cationic centers are not bound, the positive charge being detrimental, but a negative charge is not absolutely essential because some neutral forms are known to bind (Lindup, 1987).

The other drugs binding at Site II (Table 3-5) are varied in structure, and the locale of their binding has generally been implied through competition, particularly with the esterase activity of Tyr-411. Binding of 3'-azido-3'-deoxythymidine (AZT) has been localized to Site II by X-ray diffraction (He and Carter, 1992). Many of the Site-II ligands are nonsteroidal antiinflammatory agents containing ionized carboxyl groups (Li *et al.*, 1988; Wanwimolruk *et al.*, 1991).

6. *Esterase Activity*

The catalytic effect of serum albumin on *p*-nitrophenyl esters, later to be localized to Tyr-411 of HSA, was first noted in 1951 (Dirks and Boyer, 1951). At pH 7.4 the rate constant for the reaction for HSA is $10^3\ M^{-1}\ s^{-1}$, much higher than for other proteins (Koh and Means, 1979). The acetyl group is transferred to the phenolate radical and then lost slowly, but apparently more rapidly than the acetyl group from aspirin is lost from Lys-199.

Various other substrates have been found to be cleaved at this site, largely through the work of K. Ikeda's laboratory (Kurono *et al.*, 1992)—2,4-dinitrophenyl diethyl phosphate, *p*-nitrophenyl esters of alanine and glycine, and *N*-*trans*-cinnamoylimidazoles, for example. The detoxication of cyanide by reaction with elemental sulfur to form thiocyanate has been traced to Tyr-411 as well (Jarabak and Westley, 1991).

A comparative study of the nitrophenyl acetate hydrolysis by albumins of different species showed a 10-fold drop in the order human > cow > pig > rabbit > baboon > dog > snake > fish, with no activity by horse albumin (Awad-Elkarim and Means, 1988). This implies that the reaction does not have a usual physiological function, and that the catalysis by Tyr-411 is the result of a chance arrangement of nearby groups. The amino acid residues forming the binding pocket in domain IIIA are highly conserved between human and other mammalian albumins (see Fig. 4-6), but the crystallographic resolution of the conformation of this site is not sharp enough to explain the absence of activity in horse albumin (Ho *et al.*, 1993).

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An accelerated decomposition of 4-hydroxycyclophosphamide by albumin to yield phosphoramide mustard is much slower, k_{cat} = 285 M^{-1} min^{-1} (Kwon *et al.*, 1987), and has not been traced to a particular region of the molecule.

7. *Location of Site II*

The tertiary structure of the major binding pocket in subdomain IIIA, the after cargo hold of albumin, is shown as a dotted surface diagram on a ribbon model in Fig. 3-7. The residues lining the binding pocket (for triiodobenzoate) are listed in Table 2-5 and are denoted by asterisks in Fig. 2-9. In homology with those of Site I, these residues lie in domain-III helices 1, 2, 3, and 4, except that two are in helix 6. According to He and Carter (1992) the ligand site is closer to helix 1 than is the case in subdomain IIA. Tyr-411 is in this hydrophobic pocket, its phenolic oxygen atom interacting with Arg-410 and lying within 4 Å of the carboxylate of Glu-450.

From the size of acceptable ligands (e.g., LCFAs are excluded) the dimensions of the hydrophobic pocket have been estimated at 8 × 16 Å (Wanwimolruk *et al.*, 1983) and later as 21–25 Å for the long dimension (Irikura *et al.*, 1991). Access to the pocket is apparently blocked by dimerization of the albumin molecule; both the esterase action of Tyr-411 and the binding of L-tryptophan (Sollenne *et al.*, 1981) are abolished in the dimer. Hence dimerization may

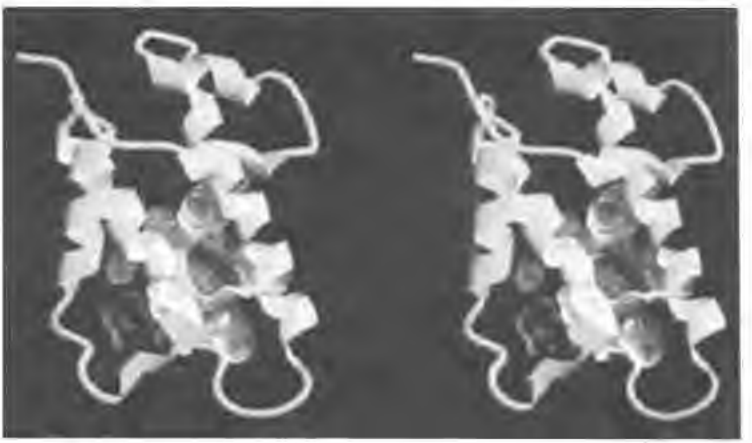


Fig. 3-7. Dotted-surface ribbon diagram of the major binding pocket inside subdomain IIIA (Sudlow Site II), with ligand. Reproduced with permission of the authors and Academic Press, from Carter and He (1994).

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appose subdomains IIIA of both molecules; if the molecules are linked by an S–S bond between the two CySH-34 residues in domain I, they would lie side-by-side in a parallel alignment.

Fluorescent energy transfer data predict a distance of only 15–17 Å from the single tryptophan (Trp-214) to a Site-II ligand (Kasai *et al.*, 1987; Irikura *et al.*, 1991), whereas the distance to a Site-I ligand is greater, 22–23 Å. The folded, U-shaped X-ray model (see Fig. 2-7) allows us to see how this is possible, the helices of subdomain IIIA being very close to those of subdomain IIA; the positions of fluorescent centers of the ligands are not precisely known, so a ligand in Site II could indeed be closer to Trp-214 than would a ligand in Site I.

Allosteric effects between these two binding sites also imply sharing of a common face. Binding of diazepam increases the $-\Delta H$ for binding of warfarin (Dröge *et al.*, 1985b), and binding of warfarin affects the NMR pattern of ^{19}F-labeled tryptophan (Jenkins and Lauffer, 1990).

Glycation of HSA, which occurs nonenzymatically *in vivo*, has been shown to affect binding of Site-II drugs but not that of Site-I drugs (Okabe and Hashizume, 1994). Because the primary site of glycosylation is Lys-525 (Chapter 6, Section II,B,3,a), a microenvironmental change in Site II was proposed.

Long-chain fatty acids, which bind first to a site in domain III distinct from the above site in subdomain IIIA, at low levels ($\bar{\nu} = 1$–3) influence the affinity for ligands in both Site I and Site II. The effect is probably coincident to the conformational consolidation by LCFAs described earlier (Section I,A). Palmitate, 1–2 M/M, enhances the first binding constant for bilirubin (Reed, 1977), chiefly through an effect on k_2, the rate of release, and palmitate or oleate increases warfarin binding as judged by CD (Sebille *et al.*, 1984). Oleate concomitantly causes a loss of some of the specificity of Site II (Birkett *et al.*, 1977), and affects the CD of both diazepam and oxyphenylbutazone (Dröge *et al.*, 1985a). The binding of steroid sex hormones is slightly (≈15%) enhanced, but estrogen binding is unaffected (Watanabe *et al.*, 1990). The precise site of LCFA binding in domain III has not yet been identified; an explanation of the manner in which the presence of a single oleate or palmitate affects so many aspects of the overall molecular structure is awaited with interest.

E. Miscellaneous Anionic and Neutral Ligands

Many compounds bound by albumin cannot be assigned to the LCFA, bilirubin-Site-I, or Site-II locations described above. For some of these the locus is known—CySH-34, for example—but for many there is little or no evidence to pinpoint the site.

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1. Ligands at CySH-34

Nitric oxide, NO, known chiefly as an oxidizing gas, has recently been identified as an "endothelium-derived relaxing factor." It has vasodilatory, antiplatelet, and neurotransmitting properties. The concentration of free nitric oxide in plasma is very low; much of the NO in the body is bound with free thiol groups of proteins as *S*-nitrosoproteins, which extends its half-life to the order of hours. Of the total NO in plasma, ≈7 μ*M*, 82% has been found to be carried as *S*-nitrosoalbumin (Keaney *et al.*, 1993). It can be detected by HPLC or GC followed by a photolysis step yielding measurable chemiluminescence. Because the complex accounts for only a little over 1% of the albumin thiol, it is not surprising that its presence has heretofore been undetected. Its concentration has been shown to vary with blood pressure changes as in hypertension and shock.

The routine presence of cysteine and glutathione as mixed disulfides on CySH-34 was noted in Chapter 2 (Section II.B.5). Exogenous substances, chiefly drugs, are also coupled in this fashion. The antirheumatic agent, aurothiomalate, apparently forms a mixed disulfide in a reversible manner, with $K_A = 3 \times 10^3\ M^{-1}$ (Shaw *et al.*, 1984; Pedersen, 1986). Mössbauer spectra and X-ray absorption studies detect no competition with the Site-I and Site-II markers, dansylamide and dansylsarcosine, and additional drug molecules may bind more weakly through bridging thiomalates.

Several other drugs bind as mixed disulfides to circulating albumin. D-Penicillamine, used to treat gold toxicity occurring in chrysotherapy as well as to remove heavy metals from the body, is a thiol that will compete for binding to the albumin SH group (Schaeffer *et al.*, 1980). Two other thiol drugs are *meso*-2,3-dimercaptosuccinic acid, a thiol chelating agent prescribed in lead intoxication (Maiorino *et al.*, 1990), and captopril, *N*-2-mercaptoethyl-1,3-diaminopropane, an antihypertensive (Keire *et al.*, 1993). Disulfiram, employed in the treatment of chronic alcoholism, is converted to diethyldithiocarbamate on binding to albumin (Agarwal *et al.*, 1983).

In the course of their detoxification some aromatic compounds form thioether adducts to CySH-34. Benzene is found as *S*-phenylcysteine in albumin and hemoglobin (Bechtold *et al.*, 1992). The widely used analgesic, acetaminophen, after metabolism in the liver, becomes linked to albumin as a thioether at the C-3 position of the drug (Hoffmann *et al.*, 1985). Another nonthiol drug, *cis*-dichlorodiammineplatinum(II), has been proposed to bind to albumin through the action of CySH-34 as a nucleophilic entering group (Gonias and Pizzo, 1983).

Albumin S–S dimers have not been reliably detected in plasma, but some other plasma proteins, usually abnormal forms, will couple through the albumin thiol. These include a cryoglobulin (Jentoft *et al.*, 1982), immunoglobulin (Ig) A forms (Tichý, 1977), a mutant antithrombin (Erdjument *et al.*, 1987), and two mutant fibrinogens (Koopman *et al.*, 1992).

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2. Pyridoxal Phosphate

A covalent adduct of pyridoxal 5'-phosphate with BSA was detected as early as 1971. The initial binding can be followed through spectral changes at 334 nm. Attachment is as a carbinolamine that converts to a Schiff base (Murakami *et al.*, 1986). By borohydride reduction of the Schiff base and isolation of peptide 182–195 the site has been identified as Lys-190 of HSA (Bohney *et al.*, 1992). In BSA the interaction differs, and the recipient lysine is Lys-221 or -224 (Anderson *et al.*, 1971). Despite the proximity of the Site-1 pocket, the action of inhibitory compounds does not clearly imply that affinity for this site is a factor in the binding. The attachment on albumin is the major means of vitamin B_6 transport; absorbed pyridoxine is converted to the 5'-phosphate before binding, and a phosphatase action converts the bound form to pyridoxal on delivery to tissues (Rose *et al.*, 1986).

3. Other Endogenous Compounds

At least three other vitamins associate with albumin in the circulation. The aquocobalamin form of vitamin B_{12} binds to BSA, apparently involving hydrogen bonding to histidine residues, which is tight enough (Table 3-1) to protect its Co(III) from reduction to Co(II) by formate (Lien and Wood, 1972). Folate binds more weakly, $K_A = 9 \times 10^2\ M^{-1}$ for HSA; about 50% of circulating folate is albumin bound (Soliman and Olesen, 1976). Ascorbate and its oxidation product, dehydroascorbate, cause a decrease in fluorescence of both tryptophan and tyrosine residues. The Scatchard plots are complex in shape (Meucci *et al.*, 1987) and the affinity constant is low (Table 3-1).

Urate ion is bound to albumin, but at such low levels that it is not a factor in urinary excretion of this metabolite even in gout. At 37° the affinity constant is about $3.9 \times 10^2\ M^{-1}$, and the proportion bound is only 24% in normal subjects and 18% in the presence of gout (Farrell *et al.*, 1971; Campion *et al.*, 1975).

4. Other Exogenous Compounds

Fluorescein, often coupled to proteins to trace their movements by its fluorescence, binds to albumin reversibly. On strong irradiation with visible light it will couple to BSA covalently; the affinity-labeled site is Tyr-137, in the ascending limb of the highly aromatic loop 3 (Brandt *et al.*, 1974).

ANS was introduced by G. Weber as a probe of albumin structure through the polarization of its fluorescence (Weber and Young, 1964a). A Scatchard plot indicated four principal sites with $nk' = 3.5 \times 10^5\ M^{-1}$ (Santos and Spector, 1972). Rapid disappearance of the fluorescence on limited peptic digestion caused Weber to propose that the dye is held in crevices between large blocks or domains of the molecule. Energy transfer from the BSA tryptophan(s) predicted

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a distance of 33 Å to the binding site (Weber and Daniel, 1966). Palmitate binding at $\bar{\nu} > 1$ depressed the ANS fluorescence; decanoate was less effective. Era *et al.* (1985) have studied the CD effects of ANS binding, particularly in relation to the **N–F** transition at acid pH, and surmised about the location of the binding site. Fragment 116–185 of BSA, containing the highly aromatic loop 3, binds ANS (see Table 2-2); this may represent merely π bonding and not an actual ligand site within the albumin molecule.

Volatile anesthetic agents bind weakly, but may affect the conformation of albumin and binding of other drugs, more at Site II than at Site I (Dale, 1986). Isofluorane ($CHF_2OCHClCF_3$) and halothane were shown by ^{19}F NMR to bind to BSA in a weak ($K_A \approx 800\ M^{-1}$) but specific manner (Dubois *et al.*, 1993). Halothane increased the binding of warfarin, and trifluoroacetate decreased both warfarin and phenytoin binding. Anazolene sodium (Coomassie Blue), a trisulfonated anilinoazo dye widely used to measure total protein (Bradford, 1976), binds to HSA at three strong sites, log $K_A = 4.7$. In the strong phosphoric acid assay reagent binding is nonspecific, and reaches 100 M/M (Congdon *et al.*, 1993), about equal to the sum of lysine, arginine, and histidine residues (see Table 2-1).

5. Peptides and Proteins

Many peptide hormones, e.g., melatonin, α-melanotropin, gastrin, and corticotropin, associate with BSA or HSA. Photoaffinity labeling suggests that corticotropin binds in domain I, peptide 1–183 (Muramoto and Ramachandran, 1981). The 12-kDa serum amyloid A (SAA) protein is also found with HSA in serum. An assemblage of peptides, including arginine vasopressin, have been isolated from commercial albumin by ultrafiltration (Menezo and Khatchadourian, 1986). When dissolved in a complex culture medium the peptides were claimed to amount to 1–2% of the weight of albumin, but from purely aqueous solution only to 0.1%, implying either increased dissociation in the presence of salts or contamination from the culture medium.

A weak association of growth hormone-releasing factor, glucagon, bradykinin, and insulin with BSA was detected by electrospray ionization mass spectrometry (Baczynskyj *et al.*, 1994). With a 9 10 molar excess of the peptide, complexes were only detected with ratios of 1–2 M/M.

Albumin binds human interferons, and immobilized HSA has been used as an affinity chromatography tool since the early stages of isolation of interferon (Carter, 1981). The association is believed to be hydrophobic in nature and to involve the first 15 amino acid residues of the interferon. Binding is stronger to immobilized albumin than to albumin in solution, and is more effective if the albumin has been defatted and the interferon is a glycated form.

A hydrophobic peptide from the human immunodeficiency virus type-1 (HIV-1) gp41 protein, residues 519–541, AVGIGALFLGFLGAAGSTMGARS,

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binds to HSA, preventing the hemolytic action of the peptide on human red cells. CD spectroscopy implies that the peptide binds largely as an α helix; results with ESR labels indicate that all but the last five residues fit into a binding pocket (Gordon *et al.*, 1993).

The powerful protease activities of cobra and rattlesnake venoms are rendered harmless to the host snake through binding to the snake's own serum albumin (Clark and Voris, 1969; Shao *et al.*, 1993). The protection is species specific, because cobra venom (a phospholipase) is lethal to a rattlesnake and rattlesnake venom (a clotting inhibitor) can kill a cobra. This intriguing and crucial (for the snake) binding activity has developed through a major change in the albumin S–S-bonded loop structure during evolution (see Chapter 4, Section III,A,4 and Fig. 4-6).

An inhibitory effect of BSA on acid deoxyribonuclease, evident at pH 4.3 (Eshima *et al.*, 1983), is probably a nonspecific ionic attraction between the acidic albumin and the highly basic nuclease.

6. *Streptococcal Protein G*

Of clinical significance is the specific binding of albumin by a protein from the cell wall of various strains of *Streptococcus*. The protein, termed protein G for the G strain of *Streptococcus* (although it also has been identified in A and C strains as well), binds albumin at one site and in most strains also binds IgG at another (Sjöhring *et al.*, 1991). It apparently has evolved as an invader–host mechanism to enable the bacterium to escape recognition by the immune system and thus to facilitate its distribution by means of the circulation.

Protein G is bound tightly by albumins of humans, rats, and mice, moderately by those of rabbits, cows, horses, and chickens, and not at all by sheep serum albumin and ovalbumin (Nygren *et al.*, 1990). The protein of *Peptostreptococcus magnus* binds human, mouse, and dog albumins, but not those of rabbits or cows (Lämmler *et al.*, 1989). A protein of *Streptococcus pyogenes*, group A, binds only mouse and human serum albumins (Widebäck *et al.*, 1983).

As obtained from strain DG-8 streptococcal membrane proteins by boiling in 0.6 *M* HCl for 5 min, strong conditions even for albumin, protein G was 30 kDa in size (Widebäck and Kronvall, 1987). The whole protein as isolated and cloned from strain DG-12 was 48 kDa, and showed an affinity constant for HSA of $5 \times 10^9\ M^{-1}$ (Sjöbring, 1992). The albumin-binding domains contain repeti tive sequences and are situated towad the C-terminal end, and the IgG-binding region lies toward the N terminus. An albumin-binding domain, ≈9 kDa, has now been cloned separately (Chakhmakhcheva *et al.*, 1992).

The only information on the region of albumin that binds to this bacterial "receptor" is that the albumin fragments that would bind were those containing

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the C-terminal domain, particularly loops 6–8 and perhaps loop 8 alone (see Table 2-2) (Widehäck, 1987). More information on this interspecies recognition site will be of interest.

II. CATIONIC LIGANDS

A. Copper(II) and Nickel(II)

Copper(II) and nickel(II) deserve special consideration among the metals because most mammalian albumins bind them more tightly and more specifically than they do other cations. Kolthoff and Willeford (1958) found that the first Cu(II) ion was not distributed among a number of loci of similar strength on BSA, but appeared to occupy a single site; this site was not the thiol group.

1. Location of Cu–Ni Site

Identification of the Cu(II) site occurred, like many findings, by serendipity. During a sabbatical visit to the Carlsberg Laboratorium in 1959, the author was testing methods for specific cleavage to isolate large fragments of BSA. Strangely, the amino-terminal aspartic residue became undetectable by FDNB even without cleavage, merely on dialysis of the albumin against water. After some puzzlement L.K. Ramachandran suggested checking the possible presence of copper in the laboratory water; at that time the Carlsberg distilled water was stored in large copper tanks. The concentration of Cu(II) in the water was found to be only about 10^{-8} *M*, but the BSA had acted as a scavenger to accumulate the metal ion. Shortly it was shown that addition of increments of $CuCl_2$ to BSA up to $\bar{\nu} = 1.0$ stoichiometrically blocked the aspartyl α-amino group to reaction with FDNB (Peters, 1960). Its binding site thus appeared to involve the amino terminus.

The isolated peptide 1–24 and even BSA peptide 1–4, Asp-Thr-Ala-Lys, bound copper as strongly as did intact BSA (Table 3-1) (Bradshaw *et al.*, 1968). The first few residues are disordered in the crystal structure and would have the flexibility to form the square-planar bipyramidal Cu(II) site. Subsequently, binding with essentially full affinity was seen with the synthetic peptide 1–3 of HSA, Asp-Ala-His-*N*-methylamide, and with near-full affinity by the generic analog, Gly-Gly-His-*N*-methylamide, showing that the only obligate amino acid species was a histidine in the third position (Camerman *et al.*, 1976). The Cu(II) ion is held tightly in a chelate ring embracing the α-NH_2 nitrogen, the nitrogen atoms of the first two peptide bonds, and the 3-nitrogen of the histidine imidazole ring (Fig. 3-8). ^{1}H NMR studies implicated Lys-4 as well in intact albumins (Sadler *et al.*, (1994). The affinity constant is so high that it is difficult to measure; reported values range from log K_A of 11.2 to 16.2 (Lau *et al.*, 1974; Giroux and Schoun, 1981; Masuoka *et al.*, 1993).

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Fig. 3-8. Molecular model of structure of Cu(II) binding site of BSA. Reproduced from Peters and Blumenstock (1967) by permission of the American Society for Biochemistry and Molecular Biology.

Similar specific binding of Cu(II) occurs with albumins of humans, cows, rabbits, rats, and others with a histidine in position three (see Fig. 4-3), but not with albumins of dogs (N-terminal sequence Glu-Ala-Tyr), pigs (Asp-Thr-Tyr) (Decock Le *et al.*, 1987), or chickens (Asp-Ala-Glu) (Predki *et al.*, 1992). Whether the specific site for copper has functional significance or is a mere chance of evolution is not clear; dogs, however, are known to be more susceptible to copper poisoning than are humans (Goresky *et al.*, 1968).

2. Properties of Albumin–Copper Complex

Much of the information about the properties of the copper complex comes from the laboratory of B. Sarkar in Toronto. Both NMR (Laussac and Sarkar, 1985) and ESR (Rakhit *et al.*, 1985) show homogeneous shifts with the affiliation of a single copper ion to HSA, in confirmation of the belief that the first copper occupies a single site. There is a clearly visible spectral shift, the blue of free copper(II) being replaced by a stronger purple color, with λ_{max} = 525 nm and ϵ_{max} = 101 L M^{-1} cm^{-1} (Peters and Blumenstock, 1967). The redness is characteristic of a 4-nitrogen ring (Nickerson and Phelan, 1974), and exceeds that of the biuret color produced by copper with whole proteins in

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strong alkali (λ_{max} = 540 nm), in which case steric effects allow an average association of only about three nitrogen atoms per copper. S-Band ESR predicts the chelate ring to contain four in-plane nitrogen atoms (Rakhit *et al.*, 1985). Bond distances from the copper atom, itself 1.0 Å in diameter, as determined by X-ray diffraction of crystals of synthetic Cu-peptides, are 2.05, 1.96, 1.95, and 1.96 Å to the four nitrogens, starting with the α-amino nitrogen (Camerman *et al.*, 1976).

NMR with ^{13}C-labeled peptides in D_2O suggests that the β-COO^- of the terminal aspartyl residue participates in the complex (Fig. 3-8), perpendicular to the plane of the nitrogen ring (Laussac and Sarkar, 1980); the sixth coordination valence of the copper(II) presumably is occupied by a water molecule. As copper binds, the loss of two hydrogen ions from the peptide bonds can be seen by titrimetry (Peters and Blumenstock, 1967). Binding is negligible below pH 5. Lau and Sarkar (1975) have measured the kinetics of transfer of copper(II) ions from complexes with free histidine and HSA; the exchange rates to and from albumin were 0.67 and 0.04 s^{-1}; ΔG, ΔH, and ΔS were estimated to be about −10, −6 kcal mol^{-1}, and 16 cal mol^{-1} deg^{-1}, respectively (Arena *et al.*, 1979). CD changes on binding are slight but are identical for the peptides 1–4, 1–24, and whole BSA (Laussac and Sarkar, 1984). Suzuki *et al.* (1989) have proposed that cysteine participates in the uptake of copper; they found that copper binds preferentially to mercaptalbumin and in time forms an albumin–copper–cysteine complex. Their findings are difficult to reconcile with much of the other work on copper uptake by albumin and the role of free histidine.

Nickel(II) binds at the amino terminus in a similar manner. The nickel ion chiefly participates in a square-planar chelate ring like copper, but about 30% of the ligand is said to be held in an octahedral structure, which is less stable (Laurie and Pratt, 1986). Nickel ion is slightly larger than the copper ion, diameter 1.1 Å compared to 1.0 Å; the complex is weaker, $K_A = 4 \times 10^9\ M^{-1}$, and copper will gradually replace nickel bound to HSA (Glennon and Sarkar, 1982). The color of the nickel–albumin complex is yellow rather than purple, λ_{max} = 420 nm, ϵ_{max} = 137 L M^{-1} cm^{-1} (Laussac and Sarkar, 1980).

The portion of copper bound to albumin is about 10% of the total in plasma, the majority being incorporated into ceruloplasmin. It is commensurate with the "easily split off" copper, i.e., released by acid conditions alone, of plasma. Its concentration is about 2 μ*M*, so that the site on albumin is only about 0.3% occupied. The plasma concentration of nickel is <0.03 μ*M*; again only a minute fraction of albumin molecules are involved in its transport.

α-Fetoprotein, with the amino-terminal sequence Thr-Leu-His, binds copper(II) about as tightly as does HSA (Aoyagi *et al.*, 1978) and yields the same 525-nm absorbance peak, with ϵ_{max} = 98 L M^{-1} cm^{-1} (Lau *et al.*, 1989). It will receive copper from albumin, and presumably has a role in handling of this ion in the fetus.

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Reviews of copper–albumin chemistry are those of Harris (1991) and Frieden (1986). The physiology of transport in the body is discussed in Chapter 5 (Section II,B).

B. Calcium and Magnesium

Careful studies of the interaction of calcium ions and serum albumin date to 1934 (McLean and Hastings, 1934), and reviews of the binding of calcium to serum proteins appeared as early as 1913 [noted in (McLean and Hastings, 1935)]. The binding is weak and concerns only 1–2 Ca^{2+} ions per albumin molecule, but is highly significant from a physiological and clinical standpoint.

As has been taught to medical students since the 1930s, about 45% of the 2.4 m*M* of circulating calcium is free, 45% bound to serum proteins, chiefly albumin, and the remaining 10% is complexed to small molecules such as citrate and phosphate. The ionic calcium could be measured only by ingenious use of the amplitude of contraction of the beating frog heart *in vitro* (McLean and Hastings, 1934) until an ion-specific electrode for calcium in blood was developed in about 1970. Ionized calcium is the optimal clinical measurement of clacium, but unfortunately is not used routinely because the temperature, pH, and pCO_2 must be carefully controlled from the instant of blood sampling, as in measuring blood gases. It is the ionized calcium concentration on which hyperparathyroidism is best diagnosed.

The pK_A for the Ca^{2+}–albumin association at pH 7.4 and ionic strength 0.15 has been reported as 2.03 (McLean and Hastings, 1935), 2.0 ($n = 12$) (Pedersen, 1971), 2.96 ($n = 1$) plus 1.9 ($n = 3$) (Fogh-Andersen, 1977), and 2.5 ($n = 1$–3); more recent values are given in Table 3-1. The effect of temperature is very slight, but the effects of pH and ionic strength (Pedersen, 1972a) are considerable; within the clinically encountered range of 7.2 to 7.6 the K_A rises nearly threefold, and the resulting changes in free calcium concentrations contribute to the languor of acidosis and the tetany of alkalosis.

Calcium binding to albumin is not specific, and its pK_A for serum globulins is 2.22 (McLean and Hastings, 1935). Because there is normally about twice as much albumin as total globulins, and albumin at pH 7.4 is farther from its isoelectric point, the bulk, about 80%, of bound calcium is found with albumin. Calculation from the concentrations of albumin, 0.6 m*M*, and bound calcium, 0.9 m*M*, and from Scatchard plot data both put the $\bar{\nu}$ for Ca^{2+}–albumin as 1.5 in plasma. Hence only the first two sites are of major importance.

Binding of calcium of HSA fragments that contain the amino-terminal region exceeds that to fragments from the C-terminal region. Adding this observation to the greater electronegativity of the N-terminal region places the calcium sites in domain

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I, where ^{1}H NMR has implicated histidine residues (Bos *et al.*, 1989b). Among natural mutants, those with substitutions in the N-terminal region (Kragh-Hansen *et al.*, 1994), including the presence of a propeptide or the 1 Asp → Val mutation, show effects on Ca^{2+} binding. Studies with Tb(III) as a fluorescent probe for the calcium site indicate that the carboxyl side chains of aspartic and glutamic acids are the major groups involved (Jin *et al.*, 1991), and that the presence or absence of a charge on critical imidazole groups may govern access or modify the stability of the chelate (Pedersen, 1972b). Of 15 pairs of adjacent Asp/Glu residues, 7 are in domain I. ΔH is zero for calcium binding, and ΔS about 14 cal mol^{-1} deg^{-1} (Jacobs *et al.*, 1971), compatible with a reaction involving chelate formation. Certainly the weak binding sites are not highly structured as are those in true calcium-binding proteins (pK_A 4–8) such as calmodulin and parvalbumins, which have octahedral or pentagonal bipyramidal sites (McPhalen *et al.*, 1991), or even the γ-carboxy glutamic acid sites in vitamin K-dependent proteins.

As the pH of an albumin solution rises from 7.4 to 8, bound calcium displaces hydrogen ions and influences the N → B transition (Chapter 2, Section II.C.1,c). A proposed explanation has been that salt bridges between imidazole and carboxyl groups are broken at pH 8, and calcium competes for binding to these carboxyls (Kragh-Hansen and Vorum, 1993).

Conformational shifts on calcium binding appear to affect two immunological determinants on HSA. A subpopulation of anti-HSA antibodies has been detected in rabbit antisera, which will bind albumin only in the presence of Ca^{2+}, 10 m*M*; that there are two such sites was derived from binding of Fab or antibody on ultracentrifugation (Frankel and Liberti, 1980).

Long-chain fatty acids, particularly the *cis*-unsaturated forms such as oleic and linoleic, increase calcium binding to HSA (Aguanno and Ladenson, 1982). The mechanism is not established, and could be due to direct binding of Ca^{2+} ions by the COO^- of bound fatty soaps or to an effect of the conformational shift caused by the first two LCFA ligands. The effect could be clinically significant in lowering ionized calcium levels in plasma incident to lipid infusions, heparin administration, or adrenalin stimulation (Zaloga *et al.*, 1987).

Magnesium is bound with slightly less affinity than calcium, pK_A 2.0 (Table 3-1). The concentration of magnesium in plasma is about half that of calcium, and again about 45% is bound by proteins, so less than one site on albumin is usually occupied by magnesium. Magnesium-selective electrodes are available to detect ionized magnesium (Frye and Lees, 1974), and the importance of measuring magnesium is increasingly being realized in clinical medicine. The site(s) and mechanism of binding have received little attention, but presumably magnesium parallels calcium in many respects and shares calcium-binding sites.

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C. Other Metals and Cationic Drugs

1. *Zinc, Cadmium, and Mercury*

Zinc(II) is both quantitatively and biologically the most important non-alkali metal transported by albumin. It acts with over 100 enzymes, including alcohol dehydrogenase and some alkaline phosphates, and of its ≈15 μ*M* concentration in plasma about 65% is loosely bound and considered to be linked to albumin (Vallee and Falchuk, 1993). Thus $\bar{\nu} \approx 0.02$ M/M would be on albumin. Of the remainder a small amount is found with transferrin and about 2 μ*M* is found with α_2-macroglobulin; this fraction is rather invariant, and increases or decreases occur primarily in the albumin-bound zinc (Foote and Delves, 1984). A major portion of the zinc of whole blood is found in the red cells, but studies with ^{65}Zn show that albumin is the main transport agent in the portal circulation.

The affinity of albumin for zinc had been considered relatively weak but more recent reports indicate pK_A values at pH 7.4 for a single site of 6.4 (Goumakos *et al.*, 1991) to 7.9 (Giroux and Schoun, 1981). The most recent value, pK_A 7.5, is included in Table 3-1. Cadmium binding is similar, with pK_A = 5.3 (n = 2). Both zinc and cadmium are believed to be chelated by imidazole and amino groups (Trisak *et al.*, 1990). NMR with ^{113}Cd suggests the first site to involve two to three histidine residues and the second to include one histidine and three carboxylates (Goumakos *et al.*, 1991). There is no competition among the first sites for zinc, cadmium, or copper. Although the second sites for zinc and cadmium may compete with each other, this is an unlikely situation except in the event of metal poisoning. Claims that both zinc and cadmium bind to thiol (Suzuki *et al.*, 1986) or disulfide (Zhou *et al.*, 1992) groups are at variance with the above reports.

α-Fetroprotein binds zinc about 3× as strongly as HSA (Wu *et al.*, 1987). This increased affinity might aid in transport of needed zinc across the placenta.

Mercury(II) added to plasma becomes 90% bound to HSA, primarily at its single thiol group. The HSA dimer and a tertiary complex of HSA–Hg(II)–cysteine can both be formed (Lau and Sarkar, 1979). Mercurial drugs are probably transported at the same site.

2. *Aluminum, Manganese, and Cobalt*

Albumin is not a major transport agent for any of these metals. For aluminum(III), transferrin has a far stronger affinity as measured by spectral changes, pK_A = 12.2, and is the major carrier. Normally total Al(III) is <0.4 μ*M* in plasma, but in patients on renal dialysis, when plasma levels can reach 5 μ*M*, the proportion on albumin reaches 34% (Fatemi *et al.*, 1991).

The K_A of HSA for manganese(II) was reported as $2.4 \times 10^4\ M^{-1}$ (Nandedkar *et al.*, 1973), but in blood it apparently also binds primarily to transferrin, in

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competition with iron(III) (Scheuhammer and Cherian, 1985). Only about 5% of added ^{54}Mn was found to be associated with albumin in plasma. Cobalt(II) binds to albumin with $K_A = 6.5 \times 10^3\ M^{-1}$ (Nandedkar *et al.*, 1974). Although it can apparently compete with Mn(II) for binding sites, ^1H NMR perturbations with Co(II) were consistent with its binding to the amino-terminal copper–nickel site of BSA and HSA (Sadler *et al.*, 1994). Its plasma level is <0.01 μM.

3. Cationic Drugs

Although albumin binds primarily to anionic drugs, Kragh-Hansen (1981) listed 11 cationic drugs that bind to HSA; examples are imipramine, chlorpromazine, and quinidine (Table 3-5). Information on possible binding sites is not available.

The glycoprotein, α_1-acid glycoprotein, or orosomucoid, generally binds positively charged drugs more strongly than does albumin—lidocaine binds with a pK_A of 4.8 to the glycoprotein and only about 2.2 to HSA (Krauss *et al.*, 1986), for example. Of 41 drugs believed to bind to orosomucoid as compiled by Lindup (1987), most are cationic, but neutral drugs such as diazepam, warfarin, and propranolol are included, and he suggests that it is time to "discard the dogma" that α_1-acid glycoprotein binds only cationic drugs.

Rivanol is used as a precipitating agent in the isolation of albumins (Chapter 7, Section I,A). As many as 17 molecules can bind to BSA with a K_A of 150. Precipitation occurs with addition of more than 7 M/M at pH 8 (Kaldor *et al.*, 1961). The binding seems to be via both hydrophobic forces and salt-type linkages.

III. ANTIBODIES: IMMUNOCHEMISTRY OF ALBUMIN

Albumin is a good antigen. Titers of 5 mg of antibody per milliliter of antiserum are readily raised in rabbits. As a result, albumin was a popular model for immunologists even before Michael Heidelberger conducted an elegant study of the precipitin reaction in 1938, which showed that the molar ratio of BSA to antibody (IgG) at the equivalence point was 4:1. Among more recent uses are the study of the kinetics of antigen–antibody reactions (Olson *et al.*, 1989), optimization of procedures for antibody production, and coupling of haptens for antibody productions, now a major industry in the field of immunoassay. The extensive reviews of the immunochemistry of albumin by Habeeb (1979) and of its immunology by Benjamin *et al.* (1984) are recommended for further background. The aim here is to present current concepts of the number and location of antigenic determinants, their chemical nature, and of the factors governing the generation of immune reactions to albumin. Clinical implications of albumin antigenicity are mentioned in Chapter 6.

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A. Number and Distribution of Determinants

An antigenic determinant is a relative concept, because what appears as foreign to one animal species can be a friendly sequence to another, and the duration of exposure to an antigen can affect the response. Immune responses are also variable among individual animals. We will consider chiefly antibodies generated in rabbits and mice.

From work by Heidelberger cited above, the number of BSA epitopes occupied at equivalence with rabbit antisera is eight, because there are two antigen-binding sites on each antibody (IgG) molecule. This is a minimal number, however, because steric considerations limit the number of large antibody molecules that can gain access to an albumin molecule in a three-dimensional precipitin lattice.

With the advent of monclonal antibodies and monovalent Fab fragments it became possible to identify particular sites by inhibition or blocking techniques that avoid the steric problems of aggregation with precipitins. Nineteen monoclonal antibodies have recognized 13 epitopes on HSA (Doyen *et al.*, 1985), and 64 have detected 13 epitopes on BSA (Benjamin *et al.*, 1985). This is consistent with the maximum number of sites predicted by precipitin analysis, and suggests that about a third of the surface of an albumin molecule is antigenic, because the average area of an epitope is about 700 Å^2. Inclusion of the propeptide, an amino-terminal hexapeptide found on newly formed albumin (Chapter 5, Section I.D), for which a unique monoclonal antibody has been reported (Oda *et al.*, 1990), would bring the total number of recognized epitopes to 14.

Siting of determinants to particular regions on the albumin molecule has largely been accomplished by showing reactivity with one or more fragments, of which a large collection is available (see Table 2-2). The first immunogenic fragment of an albumin was produced by R.R. Porter in 1957, using mild chymotryptic digestion of BSA with concurrent dialysis. A.J. Richard *et al.* (1960) next reported nine more fragments, of 3200–6800 Da. C. Lapresle, whose pioneering work at L'Institut Pasteur used an immunologist's approach, isolated a fragment, "F1," of HSA after cleavage with a splenic cathepsin that later was shown to be most of loop 9 in domain III (Lapresle and Webb, 1965). Polyclonal antibodies isolated by immunoaffinity chromatography using this fragment F1 were monospecific, i.e., they recognized only a single site on the fragment; two monoclonal antibodies were later found that reacted with the same peptide. Walker (1976b) determined the sequence of a slightly larger version of this fragment that contained two epitopes as residues 496–585 of HSA. Habeeb and Atassi (1976) found HSA fragment 377–571 (loops 7–9) to have two to four sites, which account for almost the entire antigenic reactivity of the native protein; loop 9 was later shown to contain most or all of this reactivity (Sakata *et al.*, 1979).

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Study of BSA fragments showed that only those larger than 21 kDa formed immune precipitates, but sites on the smaller ones could be detected by inhibition tests. At least six sites were distributed along the molecule, a minimum of two per domain, with those of greatest affinity being situated in domain III (Peters *et al.*, 1977; Benjamin and Teale, 1978).

Cleavage of a protein into fragments would be expected to expose new potential determinants that were hidden in the native molecule. Albumin is no exception (Ishizaka *et al.*, 1960). Denaturation by reduction (Goetzl and Peters, 1972) or other means (Maurer, 1959) revealed new antigenic sites; many of the immune complexes showed bizarre properties such as failure of expected precipitation.

B. Sequences and Chemical Groups Involved

Using the technique of inhibition by synthetic short peptides, Atassi *et al.* have predicted that five epitopes of BSA include sequences 137–146, 329–338, 527–536, 309–315 + 360–363, and 560–566 + 557–554 (Atassi *et al.*, 1979) (their residue numbering has been corrected to conform to that of Fig. 2-2). The first three of these are the tips of long loops 3, 6, and 9, respectively. The other two include noncontiguous segments in short loops 6 and 9. They were recognized by both rabbit and mouse antisera (Sakata and Atassi, 1980a). Ueno *et al.* (1994) reported transfer of a photoaffinity label from two monoclonal antibodies to the regions 299–338 and 537–554 of BSA; these locations are close to two of the five listed above, possibly coinciding considering the limited resolution of this technique.

Sites proposed by Atassi for HSA were in similar locations to those on BSA, residue numbers all being one higher owing to the deletion at 116 in BSA (Sakata and Atassi, 1980b). The sequences for HSA, in the one-letter code, are YLYEIARRHP, FLYEYARRHP, ALVELVKHKP, VESKDVC–CAAA, and ADDKETC–CKEV. The sites at the tips of the long loops are all helical regions, whereas the sites at the short loops are not (see Fig. 2-9). Significant portions lie on the surface of the molecule, as the name *epitope* implies (Carter, 1994).

Antigenic determinants usually include charged and/or aromatic residues such as Asp, Glu, Lys, Arg, Tyr, and Trp. The five sequences listed above all include Asp and/or Glu and Lys and/or Arg. Those at the tips of long loops 3 and 6 include Tyr as well. (Recall that loop 9 contains neither Trp nor Tyr.) None of the readily accessible residues, such as Lys-199 and Tyr-411 (Chapter 2, Section II,B,4), is included. The carboxylate group is of prime importance in albumin as judged by earlier chemical modification studies. Esterification of about 10% of Asp and Glu side-chain carboxyls decimated the immunoreactivity of HSA (Jacobsen *et al.*, 1972), with little effect on the tertiary structure. Amino,

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guanidine, phenol, and imidazole groups could be modified much more extensively with little immunochemical effect; 96% acetylation was needed to destroy reactivity (Habeeb, 1979).

For many years there was debate whether each of the epitopes of HSA and BSA is unique or whether there is some degree of cross-reaction among similar sites. The answer appears to be that there *is* a degree of cross-reactivity between domains, albeit of much weaker intensity than the homologous reaction (Atassi *et al.*, 1976; Peters *et al.*, 1977; Doyen *et al.*, 1985). That some investigators found cross-reactions between nonoverlapping fragments while others did not can probably be attributed to the variations in antibodies and their affinities and to the methods of detection of reactivity. A clinching finding was that some of the low-affinity monoclonal antibodies, detectable only by absorption rather than precipitation, recognize sites on different portions of the BSA molecule (Morel *et al.*, 1988). Sequence homology supports this result; although overall there is only 10–25% homology between any two domains, the predicted determinants at the tips of loops 3 and 6 of HSA, listed above, differ only in two residues.

Cross-reactivities among animal species generally correlate with differences in amino acid sequence, as is seen later (Chapter 4, Section III,A). Reactions among mammalian antigens range from 90 to 0% depending on the taxonomic proximity. They may not be reciprocal; thus, pig albumin gives 5.5 and 19.4% of the precipitin of the homologous antigen with anti-HSA and anti-BSA, respectively, but neither HSA and BSA precipitates at all with anti-pig SA. Weaker cross-reactions can be demonstrated by observing the antibodies of weaker avidity with immunoabsorption.

Antisera from closely related animals are in general more discriminating than those from distant relatives. Although mouse and rabbit antisera show the same reactivities with BSA (Sakata and Atassi, 1980a), goat antisera would be expected to react to small differences among ungulate albumins. With longer immunization times antisera tend to become less discriminating, as seen in the increasing cross-reactivity of rabbit anti-BSA with goat and sheep albumins from 7 to 400 days of injection (Sakata and Atassi, 1979).

Benjamin *et al.* (1987) have identified a monoclonal antibody, HSA-1, which is unique for HSA and unreactive with albumins of other primates; such tools will find use in forensic medicine and paleontology. At least two monoclonal anti-HSA and one anti-BSA antisera are available commercially, e.g., from Sigma Chemical Co.

Cross-reactions between albumin, transferrin, and α-fetoprotein were observed if the proteins were unfolded by carboxymethylation (Pekkala-Flagan and Ruoslahti, 1982); reaction with vitamin D-binding protein was weaker.

Cross-reactivity also occurs between albumins and unrelated proteins, some expected, some unexpected. Antisera to the rat liver enzyme, sulfite oxidase, cross-react about 20% with HSA or BSA, and 10% with the albumin relative,

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vitamin D-binding protein (Chapter 4, Section II,C) (Bellissimo and Rajagopalan, 1991), and these proteins cross-react as well with the B700 murine melanoma antigen (Gersten *et al.*, 1991). BSA and sheep albumin reacted with what appeared to be an impurity in chick ovalbumin and ovomucoid preparations (Weigle and McConahey, 1962). A mouse monoclonal antibody to pig brain tubulin, an intracellular structural protein, was found to react with reduced, immobilized HSA, but not with cow, pig, mouse, rabbit, or chicken albumin (Horejsí and Dräber, 1984). The above reactions appear to be accidents of protein evolution.

C. Albumin Immunology *in Vivo*

BSA demonstrates strong cellular immunity via T lymphocytes as well as humoral immunity via B lymphocytes. T-cell determinants have been studied with 19 monoclonal antibodies (Benjamin *et al.*, 1985). Both suppressor and helper reactions were demonstrated. Only four antigenic regions were identified; these were spread over the BSA surface, and each was found to be domain specific without cross-reaction. Their interspecies reactivities were different than those of the corresponding B-cell determinants (Weinbaum *et al.*, 1974).

BSA T-cell epitopes recognize I-A or I-E molecules of the mouse histocompatibility complex (MHC) on the surface of the antigen-presenting cells (Benjamin *et al.*, 1985). The cellular response is under Ir gene control. There is evidence that the aggregate epitopes (agretopes), which interact with the Ia molecule through T-cell responses to multiple regions, are repeated several times and hence are cross-reactive, in contrast to the T-cell epitopes themselves, which are not (Atassi *et al.*, 1982; Benjamin *et al.*, 1985).

Several laboratories have noted the variation in number and affinity of (humoral) antibodies produced at different times after primary immunization. In contrast to plateau values of antibodies to BSA fragments obtained between 15 and 398 days of immunization, antisera drawn at 7 days showed different relative antibody yields for several different fragments, and antibodies against additional weak sites on a large fragment were detectable (Sakata *et al.*, 1979). Early, but not late, antisera to native BSA were also observed to react with both native and denatured BSA (Apple *et al.*, 1984).

Relatively small fragments of BSA, e.g., loop 9, residues 506–583 (see Table 2-2), can suppress the response to whole BSA injection as studied in the laboratory of G. Michael. T-cells binding to the fragment also bind to intact BSA, but T-cells binding to whole BSA do not necessarily bind the fragment (Zhang *et al.*, 1987). An effect of idiotype recognition is seen in the action of a monoclonal anti-BSA antibody, 3C-7, to suppress the T-cell response when

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injected prior to BSA (Eddy *et al.*, 1987). Michael (1989) has also noted that tolerance to BSA can be produced by instillation of the fragment 506–583 into the ileum of mice, and that cationization (chemically adding cationic groups such as amines) of BSA or the fragment markedly alters its *in vivo* effect. Thus, cationized fragment 506–583 enhances the response to whole BSA, whereas native fragment suppresses it (Michael, 1991). This discovery has potential application to the generation of antibodies or to the prevention of anaphylaxis.

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4

Genetics: The Albumin Gene

No aspect of albumin has grown more explosively in the past decade than its genetics. Biochemists have seized on albumin as a model for regulation of gene expression in the liver, as they did earlier for studying the physical chemistry of proteins. Thanks to this interest, the complete sequence of the albumin gene, its familial relation to α-fetoprotein (AFP), to the newly discovered member of the albumin superfamily, α-*albumin* (ALF), or *afamin*, and to the vitamin D-binding protein (DBP), the sites of some 58 mutations, and the causes of the interesting condition of *analbuminemia* have all been revealed. It seems logical to consider first the location and structure of the albumin gene.

I. GENE STRUCTURE

Albumin synthesis is governed by a single-copy or unique gene (Hawkins and Dugaiczyk, 1982), which is expressed in a codominant manner, i.e., both alleles are transcribed and translated. The human albumin gene lies on the long arm of chromosome 4, near the centromere at position q11–22 (Harper and Dugaiczyk, 1983). The gene was located by use of *in situ* hybridization with radiolabeled DNA probes on karyotype preparations; the locus was narrowed to q11–13 by study of rodent–human hybrid cells (Kao *et al.*, 1982; Kumit *et al.*, 1982; Cooke *et al.* 1986). As early as 1977 the gene for vitamin D-binding protein, identified then only as Gc globulin (see Section II,B), had likewise been pinpointed to position q11–13 on human chromosome 4 by study of a child with a genetic mutation (Mikkelsen *et al.*, 1977).

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The genes for four proteins of the albumin family are now recognized to occur in tandem in the rat (Cooke *et al.*, 1987), mouse (Yang *et al.*, 1990), and humans. The α-fetoprotein gene lies 14.5 kilobase pairs (kb) downstream (in the 3'-phosphodeoxyribose direction) from albumin (Urano *et al.*, 1984) and the (rat) α-albumin gene lies 10 kb downstream from that (Bélanger *et al.*, 1994); whether the vitamin D-binding protein gene lies upstream or downstream of the other three has been elusive. The genes for the albumin family are on chromosome 14 of the rat, chromosome 5 of the mouse, chromosome 8 of the pig (Johansson *et al.*, 1992), and chromosome 6 of the chick (Palmer and Jones, 1986).

Other plasma proteins so far localized to human chromosome 4 are C3b inactivator, at 4q25, and the three peptide chains of fibrinogen, at 4q28. Plasma proteins that resemble albumin in having a transport function and being negative acute-phase reactants (Chapter 5, Section II,B) lie elsewhere, transferrin on chromosome 3 and transthyretin (prealbumin) on chromosome 18.

Two conditions show genetic linkage to albumin. One is the inherited defect of *dentinogenesis imperfecta*, in which dentin of the teeth fails to develop normally (Minghette *et al.*, 1986). The other is seen as a piebald leukodermia in humans (Yammamoto *et al.*, 1989) and as a dominant white skin color in pigs (Johnansson *et al.*, 1992).

A. Albumin Gene Sequence

The overall structure and complete base sequence of the human albumin gene as determined by Dugaiczyk and co-workers (Minghetti *et al.*, 1986) are reproduced in Figs. 4-1 and 4-2, respectively. Before delving into details of the sequence, let us consider for a moment the protein that it produces.

Although human albumin as isolated from the blood contains 585 amino acids, when first translated from its messenger RNA (mRNA) (Chapter 5, Section I,C) it also carries on its amino-terminus a 6-residue "propeptide" preceded by an 18-residue "signal peptide," both of which are removed during processing in the cytoplasm of the hepatocyte before the "mature" albumin is secreted. Thus, the translated portion of the mRNA contains condons for a total of 585 + 6 + 18, or 609 amino acids, equal to thrice 609, or 1827 nucleotides; actually the mRNA includes about 2250 nucleotides, because it has ≈400 nucleotides at its ends which are untranslated (Chapter 5, Section I,C,1,b). Yet the albumin gene contains 16,961 nucleotides, over eight times the length of the mRNA and nearly 10 times the number needed to code for the nascent albumin molecule.

The map of the gene (Fig. 4-1) helps to interpret the detailed gene sequence in Fig. 4-2. There are almost 1700 nucleotides in the 5' region to the left of the start of the transcribable gene, the capping or "Cap" site; this region will be seen

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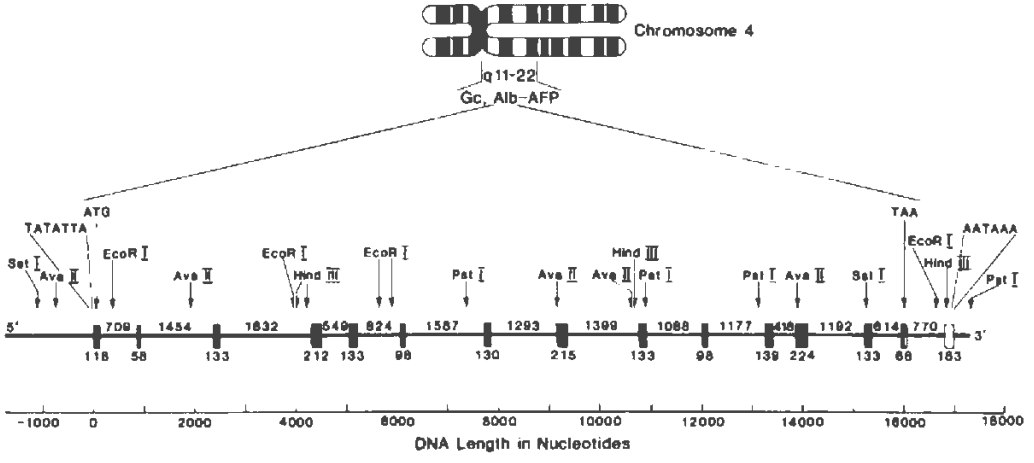


Fig. 4-1. Structure of the HSA gene. Exons are shown as boxes and intron sequences are shown as heavy lines; the numbers give their lengths in nucleotides. Solid boxes indicate the translated portions of exons. The gene begins at the Cap site of exon 1 and ends at the first polyadenylation site of exon 15. Major restriction endonuclease sites are listed above the map. Reproduced from Minghetti *et al.* (1986) by permission of A. Dugaiczyk and *The Journal of Biological Chemistry*.

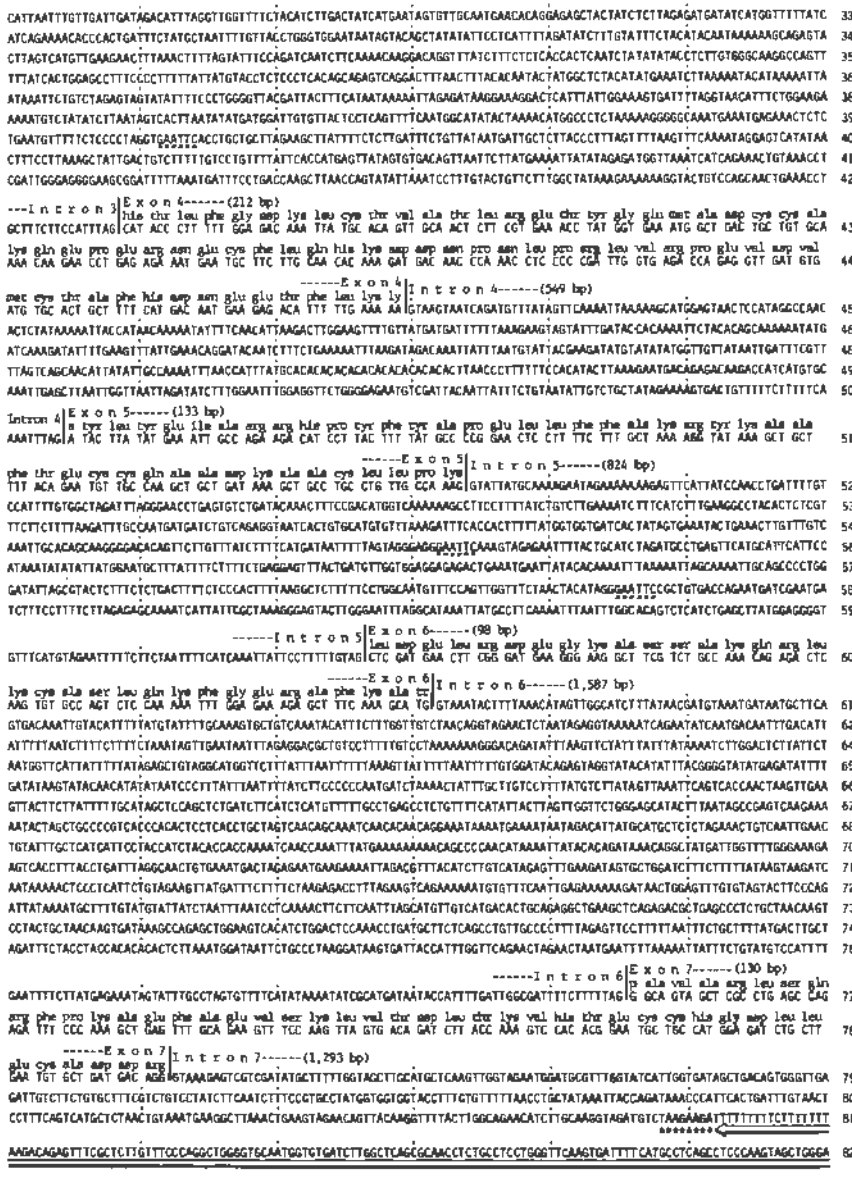
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Fig. 4-2. Nucleotide sequence of the complete HSA gene, showing 19,011 continuous nucleotides. The 14 introns and 15 exon splice sites are indicated by vertical lines, and encoded amino acid sequences are indicated above the DNA sequence. Overhead dots mark 20-nucleotide intervals. Underlines designate the CCAAT box, TATA box elements, and putative Cap site (===), the signal and propeptides and TA polyadenylation sites (--), and the *Eco*RI sites (==). Polyadenyla-

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tion sites in the 3' region are marked by a series of small circles (•). (A)n indicates the poly(A) tail. Open arrows show the locations and orientations of the *Alu* repetitive elements, and the asterisks show their flanking direct repeats. In *Alu*4 the −8 within a Δ marks a deletion of 8 nucelotides compared to the *Alu* consensus sequence. Reproduced from Minghetti *et al.* (1986) by permission of A. Dugaiczyk and *The Journal of Biological Chemistry*.

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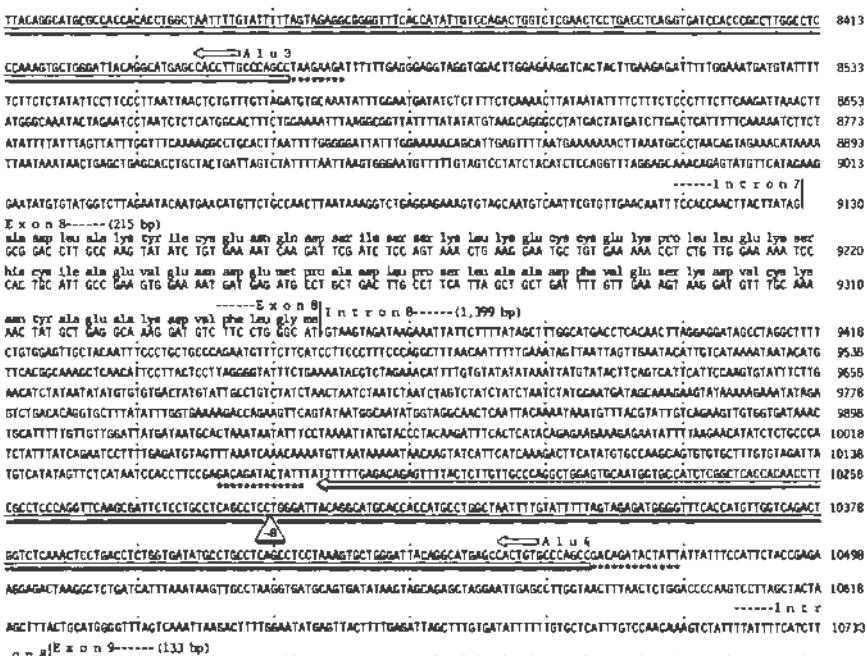


Fig. 4-2. — *Continued*

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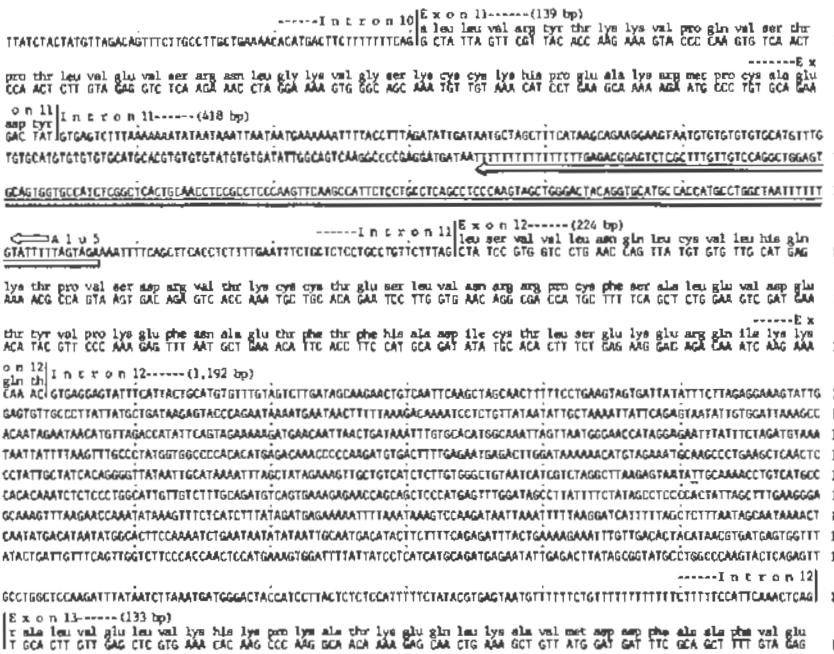


Fig. 4-2. — *Continued*

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to be important in regulating the process of transcription. The gene itself and its initial transcription product, pre-mRNA, contain 15 exons separated by 14 introns, or intervening regions, which are removed in producing the mature mRNA. This process is described in Chapter 5 (Section I).

The lengths of the 12 exons numbered 2–13 form a rough triplet pattern reflecting the three albumin domains, seen as short–medium–long–medium in Fig. 4-1 and termed A–B–C–D, in length 58–133–212–133, 98–130–215–133, and 98–139–224–133 nucleotide bases. The AB exons code for loops 1, 4, and 7, and the CD exons for loops 2 + 3, 5 + 6, and 8 + 9. (Note that this is not the functional grouping of subdomains, in which loops 1 + 2 form subdomain IA, loop 3 forms subdomain IB, etc.) Intron lengths range from 549 to 1587 nucleotides and do not appear to form any particular pattern, although the longer introns tend to occur near the middle of the gene.

At the 5' end of each of the 14 introns is the consensus sequence indicating a splice site, /GT(A,G)AGT. At the 3' end of each intron is the consensus sequence (C,T)AG/, which usually follows a pyridine-rich region. Both of these consensus sequences are in agreement with most other pre-mRNAs, including that for rat albumin (Sargent *et al.*, 1981a; Minghette *et al.*, 1986), and signify AG/GT sites of attack for RNA splicing enzymes. Each hepatocyte contains about 3000 molecules of albumin mRNA processed from pre-mRNA from a single gene; the splicing process to remove the 14 introns appears to be highly efficient.

The intron–exon splice sites show an almost perfect symmetry in the HSA sequence (see Fig. 2-1). The B–C and D–A splices occur *between codons* corresponding to homologous sites in the peptide links following the six long loops. Alternating with these, the A–B and C–D sites *split codons* between their second and third nucleotides on the ascending limbs of these long loops. One of these, the B–C splice site in loop 4, splits the tightly conserved Trp-214 residue.

Exon 1 begins with the Cap site, AGC, where transcription starts with a Gppp nucleotide. Exon 1 is only partially *translated*, beginning with the initial Met at base pair (bp) 40. In the untranslated portion, at bp −14 to −29, lies the hypothetical site for binding of 18S ribosomal rRNA to the messenger RNA (Minghetti *et al.*, 1986; Urano *et al.*, 1986). Binding sites for the H1 histone lie in the antisense DNA strand of intron 1, overlapping into the preceding exon 1 (Sevall, 1988).

Exon 14 lies at the other end of the molecule and is only partially translated, specifically its first 42 nucleotides (14 codons) immediately preceding the terminator codon, TAA. Exon 15 is completely untranslated. It does contain signal sequences, AATAAA, for polyadenylation of the mature mRNA, three in humans (Fig. 4-2) and but one in the rat. These cause termination of the mRNA about 15 nucleotides later, with addition of AAAAAAA tails, which affect mRNA stability in the cytoplasm (Chapter 5, Section I.C).

The 5' region of the gene contains numerous *regulatory elements* extending as far as −6 kb. Because these are basis of control of albumin transcription, they

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are described in the following chapter (Chapter 5, Section I,B,3 and Fig. 5-1) rather than here. *Repeat sequences*, however, are described below.

At -511 occurs a 24-fold alternating pyrimidine–purine sequence; a 32-fold similar alternating stretch lies within intron 11. Longer, *middle repeat sequences*, also occur in the albumin gene. Their prevalence may be the result of transcription to RNA, which then generates a DNA copy via a reverse transcriptase, which is in turn integrated at a new locus by "retroposition"; hence such repeating sequences are termed "retroposons" (Weiner *et al.*, 1986).

In humans the *Alu* elements, 180 to 300 bp in length, occur twice in intron 2 and once in introns 7, 8, and 11 (Fig. 4-2). These appear to be recent invaders, occurring since the mammalian radiation during evolution, and may be the cause of the human albumin gene being longer than the corresponding rat gene. The *Alu* sequences often occur in pairs in tandem, so that there are "free right" and "free left" forms.

In the rat albumin gene the short interspersed element (SINE) 4D12 occurs in intron 6 (Jose *et al.*, 1989) and a long interspersed element (LINE), L1Md, lies 961 bp downstream from exon 15 (White *et al.*, 1989). Another L1 sequence, L1Md4, appears in mouse albumin intron 12 (Bellis *et al.*, 1987). Also in the rat, 600 bp downstream in the 3' region, is found an element complementary to a small (29-bp) nuclear repeat RNA, "fr 3-RNA" (Hwu *et al.*, 1984). These middle repeat sequences have apparently invaded throughout time so that they now constitute 30 to 40% of the entire genome; they seem to have neither function nor effect.

B. Codon Usage

The *codon usage* for human albumin copy DNA (cDNA) is listed in Table 4-1. Of the amino acids having six codons, the albumin superfamily and several other plasma proteins use AGA for arginine most or nearly most frequently. Most of these favor CTG for leucine whereas albumin favors CTT; for serine, albumin differs from AFP and DBP but resembles the other proteins in favoring TCC.

Among the amino acids having four codons available, albumin, like the other plasma proteins, favors codons ending in A or T over those ending in G or C. Valine seems to be an exception. For the nine amino acids having two codons, the albumin superfamily genes and two others situated on chromosome 4, fibrinogen α and γ, invariably favor the codons ending in A or T (G + C/A + T ratio = 0.62–0.75), whereas other plasma proteins, transferrin, transthyretin, and α_1-antitrypsin, and the average human protein (2681 consolidated genes) favor C or G (G + C/A + T = 1.54) (Wada *et al.*, 1990). The selectivity toward codons means a selectivity toward the corresponding species of transfer RNA

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TABLE 4-1
Codon Usage in Human Serum Albumin as Percentage of 610 Codons[a]

TTT	Phe	71	TCT	Ser	11	TAT	Tyr	68	TGT	Cys	43
TTC	Phe	29	TCC	Ser	25	TAC	Tyr	32	TGC	Cys	57
TTA	Leu	16	TCA	Ser	21	TAA	Ter	100	TGA	Ter	0
TTG	Leu	19	TCG	Ser	11	TAG	Ter	0	TGG	Trp	100
CTT	Leu	31	CCT	Pro	42	CAT	His	69	CGT	Arg	11
CTC	Leu	9	CCC	Pro	25	CAC	His	31	CGC	Arg	4
CTA	Leu	6	CCA	Pro	29	CAA	Gln	50	CGA	Arg	11
CTG	Leu	19	CCG	Pro	4	CAG	Gln	50	CGG	Arg	7
ATT	Ile	44	ACT	Thr	24	AAT	Asn	59	AGT	Ser	21
ATC	Ile	44	ACC	Thr	28	AAC	Asn	41	AGC	Ser	11
ATA	Ile	11	ACA	Thr	41	AAA	Lys	67	AGA	Arg	48
ATG	Met	100	ACG	Thr	7	AAG	Lys	33	AGG	Arg	19
GTT	Val	26	GCT	Ala	48	GAT	Asp	69	GGT	Gly	23
GTC	Val	16	GCC	Ala	22	GAC	Asp	31	GGC	Gly	23
GTA	Val	19	GCA	Ala	27	GAA	Glu	61	GGA	Gly	46
GTG	Val	40	GCG	Ala	3	GAG	Glu	39	GGG	Gly	8

[a]Average ratio for third position: G + C/A + T = 0.62. Data from Wada *et al.* (1990).

and their respective amino acid transferases at the site of peptide assembly in the hepatocyte (Chapter 5, Section I). The significance of a codon bias between proteins made in the same cell, with presumably a common soluble pool of charged transfer RNAs, is far from clear; it may have regulatory implications.

C. Polymorphisms

The albumin gene is not invariant but demonstrates considerable *DNA polymorphism* among individuals. Dugaiczyk and co-workers found four base substitutions in the translated region (exons) of their complete gene sequence (Minghetti *et al.*, 1986) compared to an earlier cDNA sequence from another individual; three of these were silent (i.e., did not affect the amino acid selection), whereas the fourth was considered in the HSA sequence of Fig. 2-1. Comparison of their cDNA sequence with that reported by Lawn *et al.* (1981) disclosed nine different substitutions, eight of them silent. It is possible, but claimed to be unlikely, that some of these were technical artifacts. DNA sequencing by others, including the 5′ and 3′ gene regions, as well showed an

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average 1.07% substitution rate for the entire gene, with 13 variants in the coding region (Mariotti *et al.*, 1985). A discussion of phenotypic variants is given in Section IV below.

The study of restriction fragment length polymorphisms (RFLPs) has detected variant sites in the human and rat albumin gene loci. Screening the RFLP patterns produced by 27 restriction enzymes showed an average occurrence of potential polymorphic sites of 1 in 85 (or 1.18%) in humans (Murray *et al.*, 1984). In the rat up to 4% variation was observed between inbred strains (Gal *et al.*, 1985). Most of the substitutions occur in noncoding regions and so do not affect the albumin protein phenotype. Some of the useful restriction enzymes were *Eco*RV, *Sac*I, *Msp*I, *Pst*I, and *Hae*III. The *Sac*I site has been placed by Dugaiczyk in human exon 13, spanning codons 531 and 532, which is one of the silent substitutions noted by Minghetti *et al.* (1986). An *Hae*III site lies at nucleotides 8803–8816 in intron 7 (Moolman *et al.*, 1991). An *Eco*RV site lies between 0.5 and 1 kb 3' to exon 15.

II. CLOSE RELATIVES: THE ALBUMIN SUPERFAMILY

A. α-Fetoprotein

1. Structure and Properties

In 1980 Innis and Liao *et al.* both reported that the major plasma protein of the fetus, discovered in 1956 by Bergstrand and Czar and termed *α-fetoprotein* for its migration in the α-globulin region on electrophoresis, showed amino acid sequence homology to serum albumin. Although only 59 of about 600 residues were compared, the observed 40% homology was sufficient to conclude that these two proteins have a common ancestor. α-Fetoprotein, usually abbreviated AFP, had been known to resemble albumin in size (≈70 kDa), solubility, and ability to bind numerous ligands. Its physical similarity plus its reciprocal concentrations in the fetus and adult had caused AFP to be considered as a fetal counterpart of albumin. The synthesis of both AFP and albumin begins almost as soon as the fetal liver appears—15 days in the mouse, which has a 21-day gestation period—but circulating AFP levels climb to 3 g/L during human fetal life and fall to less than 5 μg/L by 1 year of life, whereas albumin levels rise only during the last months of gestation, reaching nearly the adult level of 40 g/L at birth. Human AFP is generally obtained by fractionation of cord blood or through culture of hepatoma tissue; mouse AFP can be obtained more readily from ascitic fluid of mice with certain hepatomas, and has been the source of much of our earlier information. Some reviews on AFP are those of Gitlin and Gitlin (1975), Crandall (1981), Taketa (1990), and Deutsch (1991).

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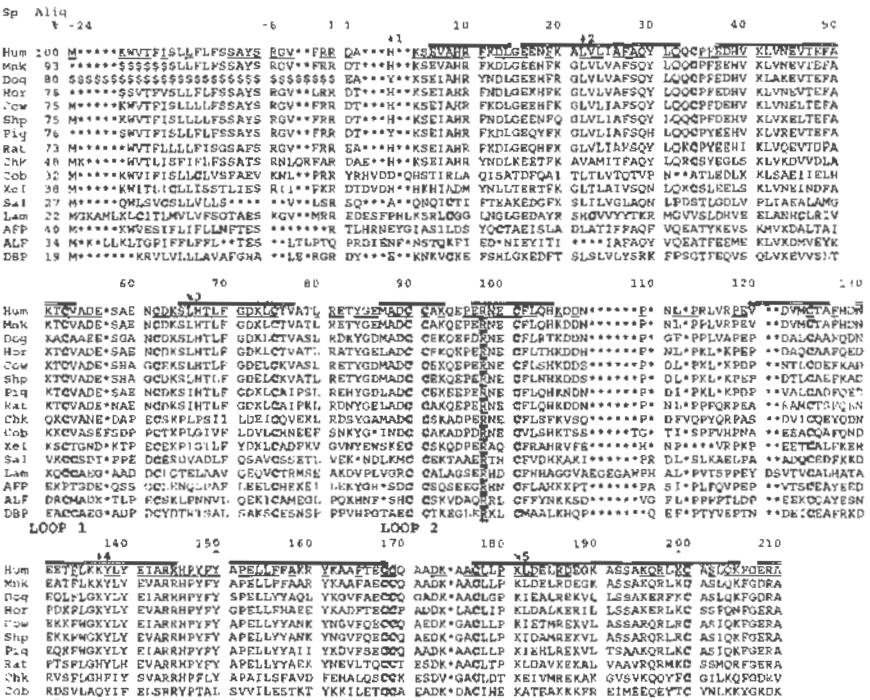


Fig. 4-3. Alignment of amino acid sequences (derived from cDNA) of 13 albumin species plus human AFP, ALF, and DBP. Alignment was generated by the ALIGN program of Myers and Miller (1988), and was altered in certain regions to bring cystine residues into phase. The abbreviations and sources are as follows: Hum, human (Minghetti *et al.*, 1986); Mnk, macaque (Watkins *et al.*, 1993); Dog (Holowachuk, 1993); Hor, horse (Ho *et al.*, 1993); Cow (Holowachuk, 1991); Shp, sheep (Brown *et al.*, 1989); Pig (Weinstock and Baldwin, 1988); Rat (Sargent *et al.*, 1981b); Chk, chicken (Cassady, 1991); Cob, cobra (Havsteen *et al.*, 1994); Xel, *Xenopus laevis* allele I (68 kDa) (Haefliger *et al.*, 1989); Sal, salmon (Byrnes and Gannon, 1990); Lam, Atlantic lamprey domains 1–3 (Gray and Doolittle, 1992); AFP, α-fetoprotein (Morinaga *et al.*, 1983); ALF, α-albumin (Allard *et al.*, 1995; Liehenstein *et al.*, 1994); DBP, vitamin D-binding protein (Schoentgen *et al.*, 1986). Align % is identity of preforms; *, inserted gaps, not included in HSA numbering; gap at position 116 in horse, cow, sheep, and pig sequences is included in numbering; $, not determined. Double lines above alignment, helical regions in HSA (see Table 2-4); ^, residues involved in ligand sites (see Table 2-5). Underlined residues denote conservation of all mammalian albumin species with K/R, D/E, F/Y, I/L/V allowed. Shaded area, Cys/2; double underline, residues conserved all forms listed. Arrows denote positions of introns (see Fig. 2-1). S–S bonded loops 1-9 are indicated.

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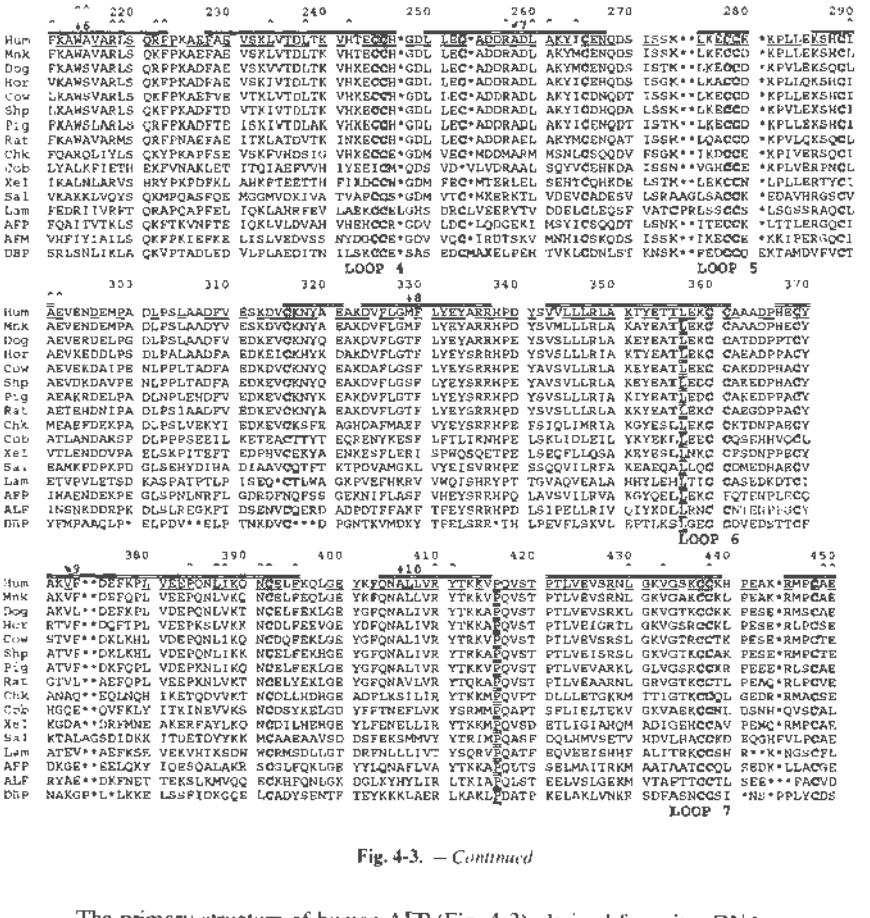


Fig. 4-3. — *Continued*

The primary structure of human AFP (Fig. 4-3), derived from its cDNA, was first obtained by Morinaga *et al.* in 1983, although cDNA of mouse AFP and much of the cDNA of rat AFP had been sequenced in 1981 (Gorin *et al.*, 1981; Jagodzinski *et al.*, 1981). The most striking resemblance is in the reproduction of the triple domain structure and the near-perfect conservation of the paired Cys-Cys residues that create the double loops of albumin.

AFP has 32 half-cystines (its amino acid composition is given later, in Table 4-5). Of these, 30 can form S–S bonds that align closely with those of HSA (Fig. 4-4). The S–S bond forming long loop 6 in HSA in missing. The half-cystine at position 18 of AFP does not readily form a homologous loop, but could pair with

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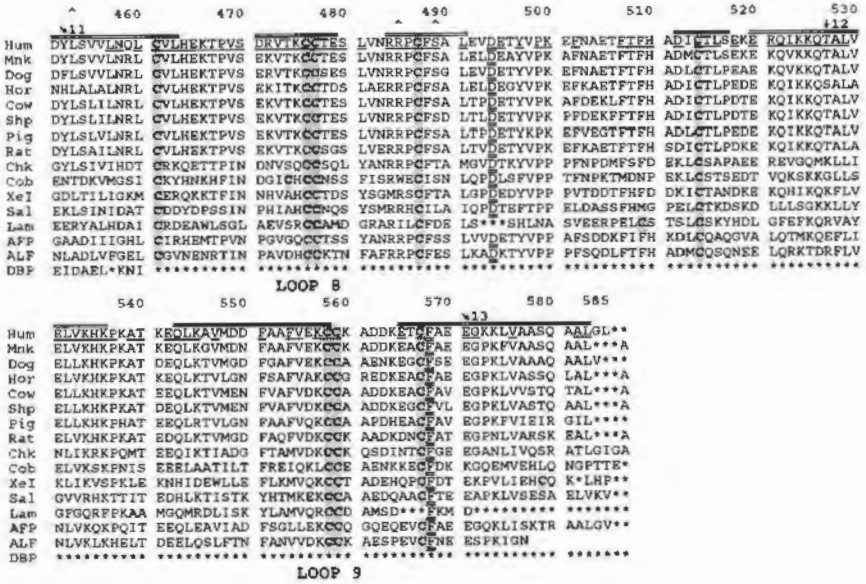


Fig. 4-3. — *Continued*

the half-cystine at position 67 to form a 49-member first loop, as shown by Morinaga *et al.* (1983). This loop would be only slightly longer than the long loops of HSA or AFP (45–47 residues), and is so depicted in Fig. 4-4, which compares the various configurations of S–S-bonded loops with evolution. Pairing of

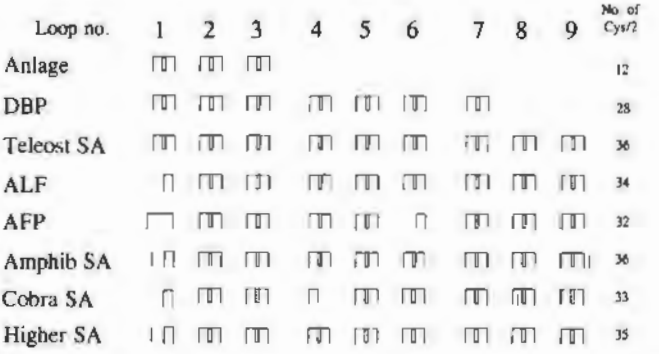


Fig. 4-4. Schematic secondary structures of members of the albumin superfamily to show the location of cysteines and disulfide bonds.

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TABLE 4-2
Features of Albumin Superfamily Structure

Protein	Number of S-S bonds	Loop changes	Number of SH	CHO[a]	Trp[b]
Early SA	18	—	0	?	?
Human DBP	14	Less loops 8, 9	0	+	1
Teleost SA	18	—	0	+	0
Human ALF	17	Loop 1A open	0	+	0
Human AFP	16	Loops 1A and 6 open; loop 1B stretched	0	+	1
Cobra SA	16	Loop 4 open	1[c]	0	1
>Teleost SA	17	Loop 1A open	1[d]	e	e

[a]CHO, Carbohydrate.
[b]Number of Trp residues.
[c]At residue 474.
[d]*Xenopus* SA (68 or 74 kDa) has additional CySH at position 577.
[e]See Table 4-7.

these residues 18 and 67 is supported by the observation that no free thiol is detectable in AFP (Table 4-2) (Wu and Lloyd, 1988).

Some doubt has arisen about the actual N-terminal residue of AFP; recently AFP isolated from a hepatoma cell culture showed Arg-Thr-Leu-His- by fast-atom mass spectrography (Pucci *et al.*, 1991), whereas the sequence derived from cDNA omitted the Arg to give an N-terminal Thr-Leu-His. It is not sure whether the difference is due to erratic processing of the precursor form by the hepatoma, resulting in retention of the Arg residue, or to incorrect assignment of the site of signal peptide cleavage when deriving the amino acid sequence from the cDNA nucleotide sequence. Strong 1:1 binding of Cu(II) by AFP (Chapter 3, Section II,A,1), however, implies that residue 3 is His and so favors the Thr-Leu-His sequence.

Homology between the HSA and AFP phenotypes (Fig. 4-3) is further apparent in the 40% identity of residues, when tested by the ALIGN program. The homology is reasonably evenly distributed through the molecule except that it is weaker (30%) in loops 1 and 2 than elsewhere (Jagodzinski *et al.*, 1981). AFP contains five more residues in the region 1–10 of loop 1 than does HSA, giving AFP a total of 590 residues compared to 585 in HSA. The Arg-Arg-His-Pro motif in loops 3 and 6 appears in AFP. A single tryptophan occurs in loop 3 of AFP but in loop 4 of HSA. Native HSA and AFP do not show immunological cross-reaction but their unfolded forms do (Ruoslahti and Engvall, 1976). Thus, homologous sequences which are buried in the native form will apparently induce antibodies when exposed to the solvent.

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Homology among the three internal domains of AFP is only 18–25%, as it is in HSA (Gorin *et al.*, 1981)—less than the 40% identity of the whole AFP and HSA molecules. Internal homology is strongest in the long loops 3, 6, and 9.

Unlike albumin, AFP is a glycoprotein. Human AFP has a single N-glycosylation site, Asn-Phe-Thr, at residues 232–234 in long loop 4 (subdomain IIA); mouse and rat AFPs each have two additional sites. The presence of a single sialic acid group confers immunoregulatory properties (van Oers *et al.*, 1989). The added 4% carbohydrate gives human AFP a calculated molecular mass of 68,950 Da. Bovine AFP has two triantennary glycans rather than one; a portion of the molecules carry biantennary chains (Krusius and Ruoslahti, 1982).

The glycan chain is a recognized source of heterogeneity. Abnormal patterns of binding to a battery of lectins such as concanavalin A form the basis of a test for hepatic cellular carcinoma (Taketa, 1990). Whether the differences are due to variations during biosynthesis or to postsecretory cleavages is not certain.

The tertiary conformation of AFP has been seen by dark-field electron microscopy at 4-Å resolution (Luft and Lorscheider, 1983). The human and bovine proteins appear U- or heart-shaped, monomeric, with outside dimensions of 80 Å. This shape (see Fig. 2-7), in contrast to the linear loop arrangement initiated by Brown (1975) for albumin (Fig. 2-1), was predicted for AFP as well as albumin by Morinaga *et al.* (1983). The absence of a disulfide bridge in long loop 6 would be expected to cause the AFP molecule to be more flexible at the "hinge" region than is albumin; some evidence of this is the ease of reduction of some of the disulfide bonds—at pH 7.5, 0.1 *M* mercaptoethanol, four thiols (cysteines) are generated in AFP but none in albumin (Wu and Lloyd, 1988). Like albumin, helicity estimated by CD is high, 67%, and is suggested to occur largely at the dense regions at the three vertices of the heart structure. Hydrophobic regions are largely protected but are exposed at extremes of pH; helicity drops to 47% at pH 2.1 (Strop *et al.*, 1984).

AFP has unique binding properties that are believed to be important in its biological function. Compared to albumin, it binds saturated and monounsaturated LCFAs poorly, but binds arachidonate and other polyunsaturated fatty acids strongly. Human AFP binds three LCFAs with log $K_A = 7.32$ ($n = 1$) and 6.72 ($n = 2$); by affinity labeling the primary site was reported to be Lys-223 in subdomain IIA (Nishihira *et al.*, 1993); in Fig 2-3 this would be residue 218. Docosahexaenoic acid (C22-6) is particularly prevalent in fetal life; its binding competes with that of estradiol by AFP of the mouse (Calvo *et al.*, 1988). [For reviews of this topic, see Deutsch (1991) and Nunez (1994).]

2. *Gene Structure*

The AFP *gene* has been mapped and sequenced for humans (Sakai *et al.*, 1985; Gibbs *et al.*, 1987), mouse (Tilghman, 1985), and rat (Nahon *et al.*, 1987).

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In humans it lies 14.5 kb downstream from the albumin gene in chromosome 4 (Urano *et al.*, 1984), and is closely similar in design. It contains 15 exons of size and internal triplet homology almost identical to those of the albumin gene (Fig. 4-1). Exon 15 is again a noncoding sequence. The nucleotides of the AFP mRNA are 52% identical to those of the HSA mRNA, although the amino acid residues of the proteins are only 40% identical (Morinaga *et al.*, 1983).

Most of the introns of the AFP gene are longer than those of the albumin gene, causing the total AFP gene to be nearly 2000 nucleotides longer than the albumin gene. Much of the increase is attributable to repetitive *Alu* and *Kpn* sequences occurring within introns (Ruffner *et al.*, 1987). The *Kpn* repeats are not found in the albumin gene, and two other repeats, *Xba1* and *Xba2* (Gibbs *et al.*, 1987), are apparently unique to the AFP gene. As with albumin, whether there is a biological function for these repeat sequences is not known.

The polymorphic frequency of the human AFP gene has been estimated as only 0.13%, or 6.4×10^{4}/nucleotide site (Gibbs *et al.*, 1987). This is almost an order of magnitude less than the 1% polymorphism observed for albumin, globin, and some other human proteins.

In the 5′ flanking region a TATAAAA TATA box ends 21 nucleotides upstream from the Cap site. There is a putative HNF1 binding region between −61 and −45, as in HSA (Gibbs *et al.*, 1987). At −69 is found a CCAAC pentamer, possibly a variant of the CCAAT "CAT box" (Sakai *et al.*, 1985). A glucocorticoid receptor (GRE) lies at −175 bp, not observed in the albumin gene. In the mouse AFP gene an enhancer region has been recognized at −2.5 and −5 kb (Camper *et al.*, 1989). The presence of this region is not required for AFP expression in heterologous cells, however. A polyadenylation site, AATAAA, begins at 126 bp within the human exon 15 (Gibbs *et al.*, 1987). Regulation of AFP expression is considered together with albumin biosynthesis in Chapter 5 (Section I.B).

B. α-Albumin

In 1994 Bélanger *et al.* and Lichenstein *et al.* each reported finding the gene for a new member of the albumin superfamily that appeared to be closely related to AFP. Bélanger *et al.* termed their protein α-albumin; I have chosen their abbreviation, ALF, for use in this book. Its gene was first found by "chromosome walking" downstream of the rat liver AFP gene; it lies at +10 kb from the AFP gene on rat chromosome 14. Shortly thereafter they used the cDNA marker for α-albumin with a human liver library to clone human ALF cDNA (Allard *et al.*, 1995). Homologous mRNAs were also detected in mouse liver (Bélanger *et al.*, 1994).

Lichenstein *et al.* (1994) termed their protein afamin (AFM), an apparent contraction of α-albumin. They first found the protein in fresh frozen human

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plasma by hydrophobic affinity chromatography, conventional salt precipitation, and gel-exclusion techniques. They then screened a human liver DNA library with an 18-mer oligonucleotide from a segment of afamin not highly homologous with human albumin, AFP, or DBP and obtained full-length afamin cDNA. By testing on a panel of somatic cell hybrids they concluded that the gene lies on human chromosome 4.

1. Structure and Properties

Human ALF, a plasma protein with a concentration of ≈30 mg/L, copurifies with apolipoprotein A-I. Its calculated p*I* is 5.65 and the calculated M_r of its peptide chain is 66,576, but its apparent M_r on SDS-gel electrophoresis is 87,000 owing to heavy glycosylation (Lichenstein et al., 1994). Its sequence shows four potential glycosylation sites (Fig. 4-3). It contains no tryptophan (Table 4-2).

Human ALF mRNA codes for a protein of 603 amino acids (including its signal peptide), or 578 residues in the mature protein (Fig. 4-3). Homology of the mature ALF amino acid sequence with the albumin superfamily is 36% to HSA, 40% to human AFP, and 21% to human DBP. The homology is strongly evident in the location of the Cys-Cys pairs and implied disulfide bonding structure, in the sequences ARRNP in loop 3 and SRRHPD in loop 6, and in the high content of tyrosine and phenylalanine in loop 3. Its disulfide bonding pattern resembles that of higher albumins rather than of AFP in the configuration of loop 1 (Fig. 4-4) and in the presence of a complete loop 6, but it is lacking the free thiol found in higher albumins (Table 4-2).

The rat ALF gene was found to be selectively expressed in adult rather than fetal liver, and not in yolk sac, brain, or kidney. ALF expression is thus more synchronous with that of albumin and DBP and apparently reciprocal with that of AFP.

2. Gene Structure

The 5′ end of the rat ALF gene was mapped for 615 bp from the initiation site (Bélanger *et al.*, 1994). It contains a TATA box and recognition sites for HNF1 and C/EBP, as does HSA. Four exons were mapped and found to match those of RSA in length.

C. Vitamin D-Binding Protein

In 1975 Daiger *et al.* identified an α_2-globulin, which had been termed group-specific component (Gc) and was used as a human polymorphic marker (Smithies, 1959), as the transport protein for vitamin D and its 25-OH metabolite in plasma. It is now commonly called vitamin D-binding protein, abbrevi-

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ated DBP, but the term, Gc, occasionally persists. The major phenotypic forms are Gc-1, Gc-2, and Gc-2-1.

The plasma concentration of DBP is about 0.5 g/L; its molecular mass is about 52,000 Da. A genetic linkage of Gc(DBP) to albumin had been recognized in 1966 through study of segregation of variants in families (Weitkamp *et al.*, 1966). [For reviews see Bowman and Yang (1987) and Cooke and Haddad (1989).]

1. Structure and Properties

Even before Gc globulin was identified as DBP, Bowman (1969) had suspected from its high cystine content and a "faint similarity" in the C-terminal sequence that it was structurally related to serum albumin. The primary structure of human DBP (Gc-1 form) was published in 1985 (Yang *et al.*, 1985) and of the Gc-2 form shortly thereafter (Cooke and David, 1985; Schoentgen *et al.*, 1986). The Gc-2 sequence is shown in Fig. 4-3. Calculated molecular mass is 51,240 Da. Sequences of rat (Cooke, 1986) and *Xenopus* (Haefliger *et al.*, 1989) DBP have also appeared.

The occurrence of paired Cys-Cys sequences and the locations of Cys residues parallel the albumin structure almost exactly, so that the proposed alignment of disulfide-bonded loops resembles that of albumin. In DBP, however, the first long double loop is closed but is half open in mammalian albumin (Fig. 4-4). The striking difference is the absence of the last two loops, loops 8 and 9 of domain III.

Amino acid homology between DBP and albumin is 19%, and between DBP and AFP is 16%; these similarities are much weaker than the HSA–AFP homology of about 40%. The parallel placement of paired Cys-Cys sequences, however, is strong evidence that DBP is a member of the albumin family. Among the domain structures of DBP, amino acid sequence identity is 19–23% and cDNA base homology is 40–42% (Yang *et al.*, 1985). As with AFP, a single tryptophan appears in loop 3 (Fig. 4-3). The albumin and AFP ARRHP motif is present only as RR (residues 336–337). Homology among the signal peptides of HSA, AFP, and DBP is about 40%, not striking for such highly conserved peptides; neither DBP, AFP, nor ALF has an obvious propeptide such as the RGVFRR sequence of albumin.

Four substitutions distinguish the DBP Gc-1 and Gc-2 forms: 152 Gly → Glu, 311 Glu → Arg, 416 Asp → Glu, and 420 Arg → Thr. Note that the net charge change from these substitutions is zero. The Thr-20 creates an O-glycosylation site on Gc-1, and it is a sialic acid group at this position that provides the electrophoretic mobility difference between Gc-1 and Gc-2. Gc-2 is reported not to be glycosylated, although both forms have a potential N-glycosylation site at 272–274, Asn-Leu-Ser.

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The tertiary structure of DBP is not known; predictions from the amino acid sequence resemble those for AFP, 53% helix and 14% β sheet. There is evidence that binding sites for different ligands lie in different domains; thus, photo affinity labeling places vitamin D binding in loop 1 in the first domain, between residues 14 and 58, whereas the binding of actin involves residues 373–405 in domain III, loop 7 (Haddad *et al.*, 1992). Crystals of the complex with vitamin D have been prepared as dimers from polyethylene glycol; they diffract to about 3 Å, and are in space group *C*2 with $a = 203$ Å, $b = 75.8$ Å, and $c = 90.9$ Å, $\beta = 109.5°$ (Koszelak *et al.*, 1985).

The affinity for 25-OH-vitamin D_3 is high, K_d = 10–100 n*M*, but DBP appears to have other functions than vitamin D transport; only 1–2% of its sterol binding site is so utilized. DBP binds long-chain fatty acids, albeit not with the avidity shown by albumin. This finding might be expected considering the loss of the primary LCFA site in loops 8–9 (Chapter 3, Section I,A,1). K_A values for palmitate and arachidonate are 7×10^5 and 6×10^5 M^{-1}, respectively (Calvo and Ena, 1989). The unsaturated LCFAs, but not the saturated ones, decrease the affinity for 25-OH-vitamin D_3 by about threefold (Bouillon *et al.*, 1992).

DBP binds the globular form of actin monomers (G-actin) with high affinity (McLeod *et al.*, 1989), preventing their polymerization; its normal loading with actin G is only about 0.05 M/M, but the loading approaches 100% when actin is released from damaged tissues. DBP is found in association with membrane-bound immunoglobulin on B lymphocytes and with the IgG Fc receptor on some T lymphocytes. One of these roles may be critical to life, because complete absence of Gc/DBP has never been noted; perhaps it is the binding of actin and its associated ADP to prevent their promotion of disseminated intravascular coagulation.

2. Gene Structure

The DBP gene has been placed in human chromosome 4, at 4q-13, by hybridization with radiolabeled cDNA (Bowman and Yang, 1987). In the linkage study with HSA, the observed recombination fraction of 0.015 places the distance from the HSA gene as 1.5 centimorgans. The defective gene causing dentinogenesis imperfecta maps 7 centimorgans from the Gc gene. Its position in relation to the HSA and AFP genes has not been established, but Bélanger *et al.* (1994) suggested that the DBP gene lies downstream of (3' to) the ALF gene owing to the synchrony in activation of ALF and DBP. The full DBP gene sequence is known for humans (Braum *et al.*, 1993b; Witke *et al.*, 1993) and the rat (Ray *et al.*, 1991). Like albumin, DBP is believed to have a single-copy gene.

The mRNA is truncated in parallel with the protein, containing but 13 exons compared to the 15 in albumin. Except for the loss of two exons the repeating pattern of exon sizes matches precisely that of HSA (Fig. 4-1) and AFP. Exon size and sequence data suggest that the loss was of exons 12 and 13 of albumin, corre-

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sponding to loops 8 and 9 of albumin (Witke *et al.*, 1993); exon 14 of albumin contains 14 residues plus the termination signal, and exon 15 is untranslated. The preservation of HSA exon 14 in DBP, however, is not readily apparent in the alignment of the amino acid sequence (Fig. 4-3).

Exon 10 (residues 373–405 in Fig. 4-3) appears to be unique, and contains the entire sequence, chiefly the link between loops 6 and 7, shown to bind actin (Cooke, 1986). The total rat DBP gene is twice the size of the rat albumin gene—35 kb—caused largely by the abnormally large introns 1, 6, 11, and 12, of 14.6, 5.2, 6.3, and 4.3 bp, respectively.

The DBP gene contains many repetitive sequences, particularly in the 5' region. In the human gene are 9 *Alu* and 7 *Kpn* elements. *Alu4*, near the 3' end of intron 8, has an unusual polymorphic poly(A) tail (Braun *et al.*, 1993a). Within introns 1, 2, and 5 of the rat gene are extensive homopurine tracts. Copolymers of AT in intron 1 and AC in intron 7 are of interest because they can lead to the formation of left-handed DNA helices (Z-DNA), implicated in the regulation of gene expression (Ray *et al.*, 1991).

Both human and rat DBP 5' flanking regions show a surrogate TATA box with the sequence TGTAAAA, at bp −29 to −23 in man. The rat region displays a putative CAT box, CCACT, ending at −87, which has not been found in the human sequence. In the 3' end of the gene, the polyadenylation signal AATAAA begins at bp 156 within exon 15. So far only two polymorphisms have been reported in the human DBP gene, using RFLP with *Bam*HI and *Pvu*II endonucleases (Bowman and Yang, 1987).

Regulatory elements of albumin, DBP, and AFP of man, mouse, and rat genes have been tabulated by Ray *et al.* (1991). All have an identified or putative site for the liver regulatory factor, HNF1, at bp −65 and −210 for human DBP. A glucocorticoid regulatory element, GRE, found in AFP but not HSA, occurs at the human DBP 5' region at −161 bp. A distal element II (DEII), found in the HSA gene at −129, is present as a putative DEII at −127 in human DBP. An enhancer region appears in the rat, but not human, sequence at −1698 bp. Function of these regulatory elements is considered in Chapter 5.

The homology among albumin, AFP, ALF, and DBP domain and exon structures strongly implies their descent from a common ancestor. A proposed chain of evolution is presented at the end of the following section.

III. EVOLUTION: ORIGINS IN THE PAST

Before searching for albumins in the circulating fluids of various species it is important to have a definition of the protein we are seeking. Solubility in pure water saturated with carbon dioxide as given in Chapter 1 can no longer suffice. Solubility in half-saturated (2.05 *M*) ammonium sulfate solutions is a possible

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definition, crude though it may be, but includes other small proteins; solubility in 40% (v/v) ethanol at pH 4.8 and −5° is more specific but not very practical.

Although albumins vary in their net charge, the ones we know are nearly always the most acidic major plasma protein, migrating the most rapidly in an anodic direction on electrophoresis at pH 8.6. They appear invariably to bind long-chain fatty acids strongly and to be of molecular mass about 67 kDa. They are high in cystine, have a free thiol form of cysteine, and lack carbohydrate.

Having at hand the amino acid and gene sequences of several mammalian albumins provides a more objective feature on which to judge a putative albumin. Paired Cys-Cys residues creating double loops are characteristic, as is a low content of tryptophan and high levels of charged amino acids (Table 2-1). The triplet structure of homologous domains implies that a molecule representing a single domain, of about 190 amino acids (molecular mass ≈22 kDa), might be found. But, as we shall see, even the triplet structure is subject to change in some lower species.

De Smet (1978) from analysis of blood sera of 416 vertebrate species found total plasma protein to become more concentrated with the change from aquatic to terrestrial life (Table 4-3), averaging about 30 g/L in fishes and amphibia and 40 to 70 g/L in reptiles, birds, and mammals, respectively. "Albumin" concentration varies with the analytical method in lower taxa, but increases even more markedly than that of total plasma protein as one ascends the evolutionary scale.

A. Identification

1. Chemical Properties

Criteria of electrophoresis, fatty acid binding, and/or size have been applied in several studies that demonstrate albumins in all mammals, birds, reptiles,

TABLE 4-3
Total Protein and Albumin Concentrations of Vertebrate Classes[a]

Class	Total protein, biuret (g/L)	Albumin	
		Salting out (g/L)	Electrophoresis (g/L)
Fishes	33.5	5	11
Amphibians	29.3	6	15
Reptiles	45.5	5	22
Birds	39.7	13	21
Mammals	69.5	33	33

[a]Data from De Smet (1978).

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Fig. 4-5. Electrophoresis of 12 vertebrate sera. (A) Pattern on cellulose acetate, pH 8.6, stained with Ponceau Red. 1, human; 2, rat; 3, turkey; 4, duck; 5, snapping turtle; 6, alligator; 7, toad; 8, salmon; 9, gar; 10, dogfish; 11, Atlantic lamprey; 12, Pacific lamprey. (B) Autoradiograph of the pattern showing the location of [^{14}C]palmitate. Reprinted from *Comparative Biochemistry and Physiology*, Volume 99B, Peters, T., Jr., and Davidson, L.K., Isolation and properties of a fatty acid-binding protein from the Pacific Lamprey (*Lampetra tridentata*), pages 619–623, Copyright 1991, with kind permission from Elsevier Science Ltd, The Boulevard, Langford Lane, Kidlington OX51GB, UK. [For a more detailed electrophoretic study of mammalian albumins, see Miller and Gemeiner (1993).]

amphibia, and bony fishes (Ambrosius, 1970; De Smet, 1978; Fellows and Hird, 1981). The mobility of albumin (i.e., the fastest migrating protein) varies among species (Fig. 4-5) and, in general, tends to be faster in higher animals. The increased negative charge that is the basis of the migration would equate with an increased Donnan effect, assisting the colloid osmotic pressure in retaining fluid within the bloodstream in the face of higher blood pressures. Turtle albumin moves so slowly that some earlier investigators claimed turtles have no albumin (Cohen and Stickler, 1958). Actually, it is present, at higher concentrations in the land tortoise than in freshwater turtles (Musquera *et al.*, 1976). Among a series of 11 mammals, albumins of the two carnivorous species, cat and dog, migrated faster than the others (Miller and Gemeiner, 1993).

Investigators have studied in some detail albumins of yellowtail carp (Yanagisawa and Hashimoto, 1984b) and other fishes (Gunter *et al.*, 1961), snakes (Masat and Dessauer, 1968; Mao *et al.*, 1985), turtles (Lykakis, 1971; Yin *et al.*, 1989), amphibians (Wallace and Wilson, 1972; Nagano *et al.*, 1973), and penguins (Osuga *et al.*, 1983). Frogs were found to have only about 0.6 g/L albumin as larvae (tadpoles), increasing some 14-fold from thyroxine stimulation (Chapter 5, Section I,B,4,b) during metamorphosis to the terrestrial adult form (Feldhoff, 1971; Nagano *et al.*, 1973).

On electrophoretic study of representative vertebrate sera (Fig. 4-5), the most rapidly migrating major serum protein was found also to be the one that

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binds [^{14}C]palmitate most strongly for mammals (humans, rats), birds (turkey, duck), reptiles (snapping turtle, alligator), amphibians (bullfrog), and teleost fishes (salmon, gar). Each of these proteins had a molecular mass of 65–75 kDa, and so filled a functional definition of a serum albumin. Fellows and Hird (1981) obtained very similar results using [^{14}C]oleate.

In recent years cDNA sequences for albumins of a reptile (*Naja*), an amphibian (*Xenopus*), a bony fish (*Salmo*), and an agnatha (*Petromyzon*) have been reported, clearly establishing the presence of an albumin homologous to that of mammals in these species. Alignment of the amino acid sequences is presented in Fig. 4-3.

In the cartilaginous dogfish the most anodic protein band did not bind palmitate (Fig. 4-5, lane 10), but a slower migrating protein of over 200 kDa did, so it would appear that dogfish lacks albumin by the above criteria. Earlier studies noted the absence of serum albumin in elasmobranchs (Irisawa and Irisawa, 1954; Gunter *et al.*, 1961; Fellows and Hird, 1981). The issue is not settled, however, because Yanagisawa and Hashimoto (1984a) reported fast-moving proteins of 70 kDa, which they believe to be albumins, in several sharks and sting rays.

Solubility characteristics of albumins also vary with species (De Smet, 1978). All chordate albumins, including that of the lamprey, are soluble in TCA–ethanol. Rivanol will precipitate vertebrate albumins and can be used for their isolation (Masat and Dessauer, 1968; De Smet, 1978). Reptilian albumins dissolve in 2 *M* ammonium sulfate (Masat and Dessauer, 1968), but not in 2.2 *M* sodium sulfite, in which only mammalian and avian albumins remain fully soluble. Heat shock, exposure to 70° for 60 min in the presence of 0.04 *M* sodium caprylate, has been effective in isolation of turtle albumins (Chen *et al.*, 1980).

2. *Biological Properties*

Binding of a long-chain fatty acid such as palmitate was noted above to be a universal indicator among candidate "albumins" of mammals, birds, reptiles, amphibians, and bony fishes, but not of elasmobranchs. Subsequent confirmation of the binding proteins as albumins by physical properties and amino acid sequences supports this use of palmitate binding as one criterion of an albumin.

The major palmitate-binding protein of Atlantic lamprey (Fig. 4-5, lane 11) was also the fastest moving major protein, but its size was markedly greater than that of mammalian albumin. Gray and Doolittle (1992) have shown that this is undeniably an albumin, albeit a most unusual one having seven rather than three homologous domains, and containing a 25-residue stretch of Ser-Thr in the fifth domain. Its sequence is considered in Section A,2 below. The major palmitate-binding band of Pacific lamprey (Fig. 4-5, lane 12) was isolated and found to be of mass 19 kDa (Peters and Davidson, 1991); it is similar in amino acid composition not to albumin but to a lipoprotein of Atlantic lamprey. The lamprey is a highly specialized jawless (*Agnatha*) species that exhibits major variations in its

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plasma proteins and a marked lipemia in its complex life cycle (Filosa *et al.*, 1986), and its genetic relationship to the fishes having jaws is obscure.

Bilirubin, likewise, has been found to bind to a strong primary site on albumins of all vertebrate classes (Fellows and Hird, 1982). In this case binding was seen even with elasmobranchs; whether this binding is to a true albumin is uncertain. Heme binding is variable. Among mammals only primates bind it strongly, yet it is bound by frog albumin.

Albumin is the chief thyroxine carrier in plasma of lower vertebrates. Binding is seen in all vertebrate classes; transthyretin appears only in birds and mammals, and thyroxine-binding globulin only in mammals (Richardson *et al.*, 1994).

Affinity for tryptophan and for certain analytical dyes generally weakens as one descends the vertebrate lineage. Tryptophan binds to mammalian and bird, but not to toad, trout, shark, or lamprey albumins, thus only to those of warm-blooded species (Fellows and Hird, 1982).

Among animal species there are widespread differences in relative affinities for organic compounds. The inaccuracies inherent in using BCG, BCP, or HABA to assay albumin in different animals are well known, as are the poor yields obtained on isolating rat or chicken albumins with Cibacron Blue (Naval *et al.*, 1982). Cibacron Blue apparently binds at the bilirubin site in HSA but not in BSA (Leatherbarrow and Dean, 1980). Only human albumin binds strongly to Affigel Blue (Cibacron Blue immobilized on agarose); one report indicates that horse and rabbit albumins will bind (Antoni *et al.*, 1978) whereas the work by Leatherbarrow and Dean contradicts this finding. In the author's experience dog albumin binds well. Bovine albumin binds poorly to the immobilized blue dye, but binds as well as does human albumin to dye in the free form; steric access may be impaired in the bovine protein (Antoni *et al.*, 1982). Addition of a long-chain fatty acid increased the affinity of HSA for Blue Dextran about 3-fold, and that of albumins of the rat, rabbit, sheep, cow, and goat about 15-fold (Metcalf *et al.*, 1981).

Dog, cow, horse, and sheep albumins bind warfarin and dansylsarcosine similarly to HSA, but rat albumin does not (Panjehshahin *et al.*, 1992). Bromphenol blue is bound by mammalian, avian, reptilian, and amphibian albumins but not by albumins of fishes. Methyl orange and HABA bind to mammalian albumins but binding is highly variable in lower species (De Smet, 1978). The esterase action on *p*-nitrophenyl acetate by Tyr-411 falls off markedly in lower species (Chapter 3, Section I,D,6). Brodersen and co-workers found that dog, pig, rabbit, hamster, rat, and cat albumins all differed from HSA in affinity for MADDS (the purported bilirubin model compound) and sulfonamides (Robertson *et al.*, 1990). Small substitutions in amino acid sequence can apparently be important in determining specificities for ligands.

The unique ability of poisonous serpents to bind their own venoms and render them innocuous to their hosts was mentioned in Chapter 3 (Section I,E,5). The major structural change effected through evolution to accommodate this binding is seen in Section III,A,4 and Fig. 4-4.

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The immunological relationships among albumins have been frequently studied, partly because albumin is a convenient model protein for immunochemists and partly because its easy isolation makes it attractive to biologists seeking to refine evolutionary pathways. The use of plasma proteins in such evolutionary studies dates from the work of Nuttall in 1904 (Nuttall, 1904).

Table 4-4 lists the degree of cross-reaction exhibited by various vertebrate albumins. Cross-reaction was about 75% with species of the same order (cow–sheep) and dropped quickly to 5–50% between mammalian orders. Interclass reactions are rare. Immunological reactivity is generally not seen in proteins with less than 30% sequence homology (Fig. 4-3 and Table 4-6 below). The author has noted, for instance, that rabbit antiserum to salmon albumin shows no cross-reaction with lamprey albumin or those of higher animals in precipitin testing. Note that weaker reactions can be detected using immunoabsorbents than by precipitation, chicken albumin showing 18% cross-reaction by this method (Table 4-4), but only 3% by precipitation with mouse antiserum (Dietrich, 1968).

Although evolutionary relationships can now be more securely traced through cDNA and gene sequence homologies (Section III,A,4), careful analysis of immunochemical cross-reactions has been useful, particularly in the hands of the late Allan C. Wilson and collaborators of Berkeley. In many of their contributions they have studied amphibians: toads (Maxson and Szymura, 1979), lungless salamanders (Maxson and Maxson, 1979), tree frogs (Maxson and Wilson, 1975), *Xenopus* (Bisbee *et al.*, 1977; Graf and Fischberg, 1986), and *Rana* (Wallace *et al.*, 1973). They have shown that albumin in frogs has continued to evolve as rapidly during the 200 million years following their differentiation as it has in more recently developed species. The result is that the serological differences between albumins of widely diverse frog species are as great as those between frogs and other orders, even mammals, and are much greater than dissimilarities among (the more recently developed) mammalian orders. Thus, the "albumin clock" (Carlson *et al.*, 1978) keeps ticking, and elapsed time is as responsible as phylogenetic differences in determining differences among proteins.

From studies of turtle relationships (Chen *et al.*, 1980; Yin *et al.*, 1989) it has been proposed that modern turtle species diverged only between 16 and 65 million years ago. Another study proposed that the very thin-snouted gavials in India belong with the Crocodylidae family rather than comprising a separate family in the order Crocodylidia (Hass *et al.*, 1992). Prager and Wilson (1976) published a broad study of genealogy of birds based on albumin, transferrin, and ovalbumin. Albumins of birds react with anticrocodilian albumin, but not with antisera to other reptilian albumins, such as lizards and turtles (Gorman *et al.*, 1971), an inheritance, perhaps, of the evolution of birds from dinosaurs.

Albumin serology places pinnipeds (seals and sea lions) near the canine carnivores (Canoidea) and distinct from the cat order (Feloidea) (Sarich, 1969). In

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TABLE 4-4
Immunological Cross-Reactions of Albumins with BSA[a]

Albumin species	Precipitin reaction[b]		*In vivo* PCA[c]	Bound to absorbent[d] (%)
	Precipitate (%)	Equivalent ratio		
Cow	(100)	5.5	++++	(100)
Sheep	75	4.7	++++	75
Goat	—	—	.	83
Pig	32	4.8	++	76
Horse	12	5.5	++	56
Human	15	4.5	++	41
Dog	14	3.5	+	—
Cat	25	—	—	—
Hamster	14	4.0	—	—
Rat	14	3.7	—	—
Mouse	10	4.4	—	—
Guinea pig	7	3.4	-	—
Valleroo	6	—	—	—
Chicken	18	-	—	—

[a]With rabbit antiBSA, 2–12 months of immunization. Similar results were obtained using anti-HSA.

[b]Percent of precipitate mass at equivalence and ratio of precipitate mass to antigen mass (Weigle, 1961).

[c]Passive cutaneous anaphylaxis, 100-μg test dose (Weigle, 1962); ++++ = maximal response.

[d]Percent of antibodies bound to fixed antigen (Sakata and Atassi, 1979).

the evolution of primates, albumin immunological distances suggest that humans diverged from New World monkeys, Old World monkeys, Asian apes, and African apes approximately 50, 30, 8, and 5 million years ago, respectively, and that humans are more closely related to the chimpanzee than is the orangutan (Sarich and Wilson, 1967).

Antigenic sites of albumin are sturdy enough to survive in fossils. Well-preserved albumin has been identified in the thigh muscle of a baby mammoth frozen for 44,000 years in the Siberian tundra. Immunologically it was shown to be as closely related to the modern Indian and African elephants as these two existing species are to each other (Prager *et al.*, 1980). Human albumin has been detected immunologically in ancient human bones dating back to the Bronze Age (2200 BC) (Cattaneo *et al.*, 1992); this capability will aid archaeologists in differentiating human and animal bones. Even in prehistoric humanoid fossil

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bones, including *Australopithecus robustus* from Ethiopia, a protein reacting like human albumin was found (Lowenstein, 1981). The albumins of Australian and South American carnivorous marsupials, including a museum specimen of the extinct Tasmanian wolf, have been tested to clarify their lineage (Lowenstein *et al.*, 1981). More recently, the rate of loss of immunological activity of albumin with time is being studied in the urinary "middens" of fossil pack rats as an aid in paleontological investigations (Lowenstein *et al.*, 1991).

3. Composition of Albumins of Different Species

Table 4-5 lists the amino acid compositions of 16 albumin species: 8 mammals, 1 bird, 3 reptiles, 2 amphibians, 1 teleost, and 1 cyclostome. Other members of the albumin superfamily, AFP, ALF, and DBP, are included for comparison. Compositions of human, bovine, and rat albumins were given in Table 2-1 and are not repeated here. Only one allele of a taxa (*Xenopus*, *Salmo*) is included; the other alleles are highly similar. Where possible, data are taken from cDNA sequences because these are more reliable than data from amino acid analysis of hydrolyzates. Thus, the "Ave. SA" figures are the average of the 13 albumin species with known cDNA sequences.

The "Ave. SA" by comparison to the "Avg. Protein" of Table 2-1 shows a paucity of tryptophan, glycine, serine, asparagine, and proline, and an abundance of glutamic acid, cystine, lysine, and leucine. Monkey albumin is striking in having but one isoleucine and the dog but five, replaced chiefly by alanines rather than leucines. Pig albumin, with 23 isoleucines, is at the other extreme for a mammal. Glycine is between 13 and 24 residues per 585 total residues except for the turtle; it is possible that the turtle samples assayed were very small, which in the author's experience tends to enhance glycine contamination. Horse and pig albumins have no methionine, and rabbit and frog have but one.

A few trends may be detected as one descends the evolutionary tree, comparing lower vertebrate species with mammals. Isoleucine tends to rise and alanine to fall. Tryptophan is highest in *Xenopus* and is lacking in salmon and chicken; a survey showed it also to be lacking in duck and turkey albumins (Feldhoff and Peters, 1976).

Neither cDNA sequences nor amino acid analyzers differentiate cysteine from half-cystine. Mammalian albumins generally show 0.5–0.8 SH/albumin by thiol measurements if freshly prepared. Fantl (1972) found bird albumins to have only about 0.2, reptiles 0.2 (with some exceptions as high as 1.1), amphibia 0.5, and teleosts 0.15 M/M. The tendency is toward higher detectable SH/albumin with later species. Teleost albumin apparently contains no unpaired half-cystines (Table 4-2, Fig. 4-4).

Carbohydrate is generally considered to be absent in a pure albumin, but nature is not so rigid when lower species are considered. Yellowtail carp, a teleost, was reported to have 12–20% carbohydrate in a 76-kDa albumin (Yanagisawa and

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Hashimoto, 1984b), and one of the two forms of *Xenopus* albumin is glycosylated, as is lamprey albumin. Albumins of mammals do not contain carbohydrate, and the molecular weights of reptilian and avian albumins of ≈67 kDa make it likely that they do not, either.

4. Sequences and Structures

The derived amino acid sequences of the 13 albumin species with known albumin cDNA sequences are compared in Fig. 4-3. Human AFP, ALF, and DBP are included for comparison. Only one allele of a species is listed, and only the first three of seven domains of lamprey albumin. Cys-Cys pairs are matched throughout, and the triple-domain structure remains apparent.

Homology of the sequences by pairwise matching for identity is shown in Table 4-6. Results follow predictions from evolutionary information—the two primates (man and macaque) and the two artiodactyla (cow and sheep) agree with each other within about 93%; between mammalian orders identity is 70–80%, between mammals and the amphibian it is about 38%, versus the teleost 28%, and versus the lamprey only 19–22%. The rat appears slightly isolated from the other mammals. The cobra sequence is an exception, having less homology to higher forms than has the amphibious *Xenopus*; this discrepancy is an example of divergent evolution to generate its venom-binding site (Shao *et al.*, 1993). Studies of albumins of other poisonous reptiles should be of great interest.

The adjacent Cys-Cys pairs, while characteristic, are not unique to albumin, appearing also in insulin, the γ chain of fibrinogen, the γ chain of immunoglobulin, a snake neurotoxin, and other proteins (Brown, 1976). The first of the eighteen S–S-bonded loops, loop IA, shows systematic variation (Fig. 4-4); it is a closed S–S loop in DBP and albumin of the salmon and, possibly, the lamprey; it contains one half-cystine (as CySH and mixed disulfides) in higher animals (Brown, 1976) with the exception again of the cobra, and is absent in ALF and AFP.

Certain other locations in addition to the cystines are highly conserved. Arg-98, Leu-357, Pro-416, Asp-494, and Phe-568 are invariant in all of the sequences shown. In the serum albumin sequences Arg-(−1), Leu-42, Pro-147, Pro-339, Phe-403, Tyr-411, and Gln-417 are preserved. At 25 other sites in serum albumins the amino acid is identical except for a single species. Of the 25 sites 7 are leucine, 3 are lysine, 2 each are aspartic or glutamic acids, tyrosine, phenylalanine, proline, or arginine, and 1 each alanine, glutamine, or glycine. Predictably, the nonconforming species is the lamprey in 14 of the 25 sites, whereas in 4 sites it is the cobra or the chicken, and in 3 sites the salmon. Conserved regions in mammalian albumins (underlined in the top line of Fig. 4-3) are well distributed through the molecule. Sequences around prolines 147, 224, 339, 421, and 537, at the tips of the long loops 3, 4, 5, 6, 7, 9, respectively, are relatively constant. Some poorly conserved and apparently less critical

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TABLE 4-5
Amino Acid Composition of Albumins and Related Proteins[a]

Amino acid	Number of Residues[b]																Avg. SAc	sdc	AFP	ALF	DBP
	Mnk	Dog	Hor	Shp	Pig	Rab	GnP	*Mus*	Chk	Alg	Ttl	Snk	Frg	Xel	Sal	Lam					
Ala	68	57	57	50	50	56	57	51	42	45	41	34	43	28	60	56	51.6	10.7	50	28	30
Cys	35	35	35	35	35	35	35	35	35	35	35	33	35	36	36	35	35.0	0.7	32	34	28
Asp	35	36	41	44	37	—		—	41		—	30		40	47	29	37.5	4.9	23	33	26
Glu	60	62	58	56	61	—		—	50		—	63		64	45	53	57.7	5.1	57	54	43
Phe	31	30	30	28	29	24	28	25	28	25	26	24	21	27	20	25	27.4	2.9	29	38	18
Gly	13	23	18	17	16	19	19	17	24	22	33	16	20	14	21	24	17.8	3.7	26	15	13
His	16	11	19	18	18	21	19	16	13	22	21	20	23	31	22	30	18.9	5.4	16	13	6
Ile	1	5	14	13	23	15	16	10	33	31	31	33	28	25	23	19	17.2	9.2	32	27	8
Lys	59	56	60	60	57	59	57	48	47	62	43	53	62	55	52	26	53.5	8.4	41	49	43
Leu	62	63	65	61	62	61	59	63	40	45	53	53	52	54	44	54	56.6	7.0	57	49	53
Met	9	4	0	4	0	1	5	7	19	8	10	7	1	12	20	10	7.8	5.8	8	8	7
Asn	17	14	11	14	13	—	—	—	16	-	—	29	—	24	6	12	15.9	5.4	19	33	18
Pro	26	28	30	28	30	26	29	31	26	32	31	24	24	33	25	17	26.8	3.7	21	28	26

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Gln	20	20	17	19	20	—		—	34	—	—	20	—	22	23	23	21.8	3.9	40	27	12
Arg	21	24	22	22	26	23	23	22	25	26	21	15	22	17	16	38	22.9	5.3	21	24	13
Ser	24	28	30	25	23	28	25	26	38	27	30	42	24	25	39	50	30.8	8.0	36	34	42
Thr	24	25	28	31	26	29	28	40	22	26	29	40	21	31	35	35	30.2	4.8	35	31	31
Val	43	41	30	36	33	37	31	33	36	27	37	33	30	24	43	36	35.9	5.0	29	36	24
Trp[d]	1	1	1	2	2	1	3	1	0	1	1	1	2	4	0	3	1.5	1.0	1	0	1
Tyr	19	21	17	20	22	22	18	21	20	16	14	20	16	16	13	10	18.2	3.2	17	17	16
Asx	—	—	—	–	—	53	47	55	—	57	50	—	55	—	—	—	–		–	—	—
Glx	—	—	—	—	—	75	86	83	—	78	79	—	96	—	—	-	—	–	—	—	—
Total	584	584	583	583	583	585	585	584	589	585	585	590	585	582	590	585	585.0	—	590	578	438

[a]Mature albumin forms, calculated to the total value shown; note that lamprey actually has a total of 1394 residues.

[b]References: Values showing Asp, Glu, Asn, Gln were from cDNA sequences (Fig. 4-3); values showing Asx, Glx were from chemical analysis of acid hydrolyzates as follows: rabbit (Rab) (Jacobs and Koj, 1969), guinea pig (GnP) (Benjamin and Weigle, 1971), mouse (*Mus*) (MacLaren and Petras, 1976), alligator (Alg) (Wallace and Wilson, 1972), turtle (Ttl) (avg. of 9) (Yin *et al.*, 1989), frog (Frg) (avg. of 2) (Wallace and Wilson, 1972).

[c]Cys/2 fixed at 35 unless a different value is known from cDNA sequence, due to losses on analysis.

[d]Trp values not given in references were taken from Feldhoff and Peters (1976).

[e]Only 12 serum albumin (SA) values based on cDNA sequences were averaged, including human, bovine, and rat from Table 2-1. sd, Standard deviation.

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TABLE 4-6
Percent Pairwise Sequence Identities of Mature Albumins of Different Species[a]

	Mnk	Dog	Hor	Cow	Shp	Pig	Rat	Chk	Cob	Xel	Sal	Lam[b]
Human	93.2	79.3	76.2	75.6	74.7	75.0	73.2	46.7	31.3	37.9	26.9	21.2
Monkey		80.3	77.7	76.5	74.8	75.2	74.0	46.2	30.8	37.8	26.7	21.2
Dog			76.2	76.0	77.6	77.6	72.3	46.0	31.3	36.3	27.4	27.4
Horse				74.1	75.6	76.2	72.6	43.1	29.8	38.4	29.3	19.6
Cow					91.9	79.6	70.0	43.8	29.4	37.4	28.3	20.7
Sheep						77.7	69.5	44.0	29.3	37.4	27.7	20.2
Pig							72.4	42.8	29.4	37.9	28.3	21.2
Rat								44.3	30.5	38.3	27.1	22.2
Chicken									29.1	37.4	27.6	19.6
Cobra										29.3	25.5	19.6
Xenopus I											26.4	18.8
Salmon												21.4
Lamprey[b]												

[a]Mature albumin sequences obtained from Fig. 4-3 were compared by the ALIGN program (Myers and Miller, 1988) modified in the FASTA package by W.R. Pearson, University of Virginia.

[b]Mature albumin of domains 1–3 only of lamprey.

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areas in mammalian albumins are residues 58–61, 121–134, 432–467, 503–506, and 578–581.

The residues involved in the two binding pockets of HSA are well conserved in the mammalian species listed, but not, in general, below that. For both Site I and Site II all of the 16 residues listed in Table 2-5 and marked in Figs. 2-9 and 4-3 are identical or have very conservative substitutions (Arg/Lys, Tyr/Phe, Leu/Ile/Val) among mammals, except for four residues in Site I, which differ in rat albumin—residues 198, 219, 242, and 264. In the submammalian albumins, Site-II residues are more highly conserved (61%) than those of Site I (38%).

Codon usage, although not shown here, indicates a marked evolutionary trend. *Xenopus* albumin, like that of man, favors AGA for arginine (Wada *et al.*, 1990), but lamprey favors CGC (Gray and Doolittle, 1992), and both of these species favor CTG for leucine whereas human albumin favors CTT. The trend is seen strongly in the average usage for the third codon position: G + C/A + T ratio is 0.62 for human albumin, 0.91 for the frog, and 1.88 for lamprey, resembling more the average of all known protein usage of 1.54. The ratio for all lamprey proteins, on the other hand, is even higher, 5:1, and for lamprey apolipoprotein I is 20:1.

Tertiary structures of the four mammalian albumins as seen by X-ray diffraction are similar. In lower albumins, although it has not been measured, helical content can be estimated from amino acid sequence comparison to be similar to that of mammalian albumins. Thus, the program of Garnier *et al.* (1978) predicts helicity of 77, 67, 58, and 60% for human, bovine, amphibian, and salmon albumins, respectively. The predicted locations of the helical regions are similar; the submammalian albumins resemble that of the cow more than of humans in that the random-chain links between subdomains IIA and IIB and subdomains IIIA and IIIB are called helical in humans but tend to be β forms in the other three species. For the other superfamily members, estimated helix of human AFP, ALF, and DBP is 53, 48, and 53%, respectively.

Table 4-7 summarizes the distinguishing features of the albumin superfamily at the present state of knowledge:

1. Albumin occurs as far down the evolutionary scale as the teleosts and lampreys. In elasmobranchs it is uncertain. DBP, the oldest of the family, appears in teleosts and above but not in lamprey or elasmobranchs; it therefore coincides with the appearance of bony tissue. An α-fetoprotein has been reported in fetal sharks, birds, and mammals, but was not found in *Xenopus* larvae, creating an apparent gap in the family lineage. ALF is so far recognized only in mammals.

2. Known albumins, ALFs, and AFPs have three domains except lamprey albumin, which has seven; DBPs have 2.5 domains. Homology between domains within these proteins is less than the homology between proteins. The highest variability is in domain I.

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TABLE 4-7
Occurrence of Albumin Superfamily[a]

Species	DBP[b]	ALF	AFP	Serum albumin	CHO	Trp
Lamprey	No	—	No?[c]	Yes	Yes	8
Shark	No		Yes[d]	No?	—	—
Salmon	Yes	—	?	Yes	Some	0
Amphibia	Yes	—	No[e]	Yes	Some	2-4
Reptilia	Yes	—	?	Yes	No	1
Birds	Yes	—	Yes[f]	Yes	No	0
Mammals	Yes	Yes[g]	Yes	Yes	No	1
Artiodactyla	—	—	—	—	—	2

[a]CHO, Carbohydrate; Trp, number of Trp residues.
[b]Hay and Watson (1976).
[c]Gray and Doolittle (1992).
[d]Gitlin *et al.* (1973).
[e]Haefliger *et al.* (1989).
[f]Lindgren *et al.* (1974).
[g]Lichenstein *et al.* (1994).

3. The DBP gene is predicted to have split away from a common DBP/AFP/ALF/SA precursor 450–600 My ago (Haefliger *et al.*, 1989; Gray and Doolittle, 1992).

4. Characteristically the albumins have ≈65% α helix; AFP, ALF, and DBP have ≈50%.

5. Large loop 1A is missing in ALF and AFP, and is replaced by a single CySH in animals above the teleosts, the cobra excepted (Fig. 4-4).

6. DBP, AFP, ALF, and lamprey and teleost albumins contain carbohydrate, as does one of two forms of the amphibian *Xenopus* albumin (Table 4-7). Albumins of higher species appear not to contain carbohydrate.

7. Mammalian albumins contain one tryptophan except that artiodactyla (pig, sheep, cow) albumins contain two (the value of 3 Trp for the guinea pig in Table 4-5 is questioned). Birds have none, like the teleost, whereas reptiles and amphibians have one to four (Tables 4-5 and 4-7). AFP and DBP contain one each, but located in loop 3 rather than loop 4. ALF has none.

8. A characteristic gap appears at residue 116 in albumins of the hoofed mammals, horse, pig, sheep, cow. These plus the albumins of rat, mouse, and dog lack the C-terminal residue.

With this information we will in the next section try to predict an evolutionary path of the albumin family.

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B. Possible Origins

Where, then, are the origins of albumin? In what form was its ancestor? Of how many domains or loops? What was its aboriginal function? What were its relationships to other early proteins?

Sequence comparisons of human albumin with the entire protein library, to answer the last question, imply a slight kinship to the paired CysCys-containing proteins listed above, and to the hemoglobin α and β chains, to complement C4A, neurotensin, α-casein, and the serum basic protease inhibitor. An immunological relationship of HSA and DBP with a mouse melanoma antigen was described in Chapter 3 (Section IV,B). Carter and Ho (1994) discussed a similarity of the h1–h4 helices of HSA to helices EFGH of hemoglobin, but concluded that it is more likely the result of convergent evolution.

Albumin appears *not* to be related to some proteins that *a priori* might have been considered as kin. Among these are the large family of intracellular calcium-binding proteins, the parvalbumins of muscle, and the lactalbumins of milk. These proteins may be examples of convergent evolution of similar functions.

Figure 4-6 is an attempt at this stage of our knowledge to derive an albumin superfamily tree. Readers should realize that information in this area is expanding rapidly and should check the latest literature if they have more than a passing interest.

The scheme incorporates the information summarized at the end of the previous section, and draws heavily on the analyses by Gray and Doolittle (1992). The suggested times of the different radiations are indicated and accord reasonably well with those predicted from fossil studies. The cyclostomes, hagfishes, and lampreys diverged about 500 My ago. The size or even existence of albumin in hagfish is not known; the seven-domain lamprey albumin is suggested to have originated from a single-domain ancestor before the gene replication events occurred to form the familiar three-domain structure. The basis for this choice is that the seven domains of lamprey show little relation among each other, and can be considered to have changed markedly during the long developmental period. In fact, albumin is one of the most rapidly changing proteins, having substituted 70–80% of its residues in 500 My, whereas retinol-binding protein, for example, changed only 30–50%, and histones less than 10% (Doolittle, 1992).

Evolution of the triple-domain structure shortly (evolutionally speaking) after the cyclostome radiation is suggested by relationships among domains. Sequence homology of a domain is greater *between species* than it is *intramolecularly* (McLachlan and Walker, 1977). Thus, human albumin domain III is 40% identical with rat albumin domain III and 28% identical with salmon domain III, but only 20% identical with human domain I. Salmon albumin domain III is only 15 and 18% identical with its own domains I and II, respectively.

Rather promptly after the triplication event DBP is believed to have evolved, losing the internal exons 12 and 13 coding for loops 8 and 9 in its

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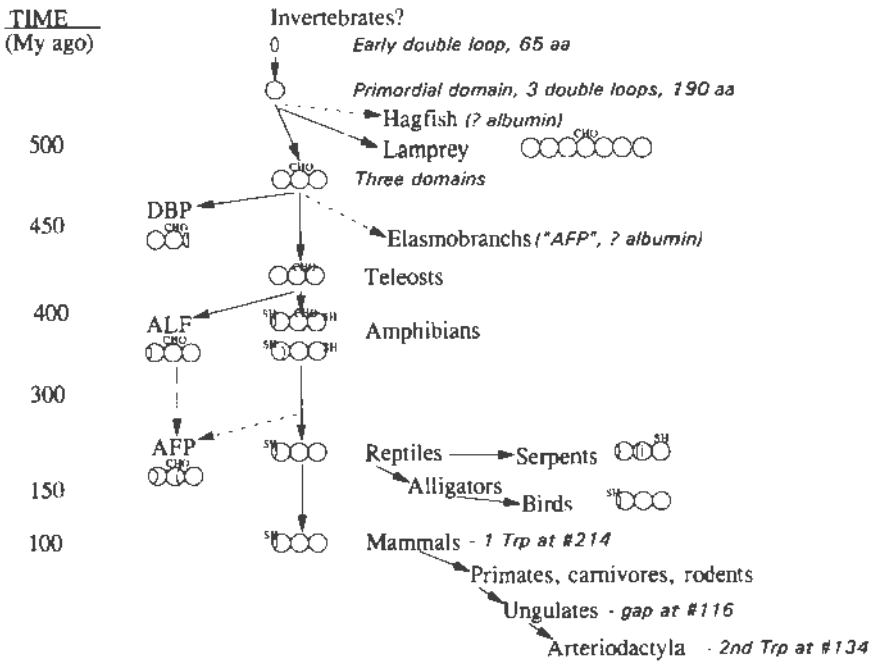


Fig. 4-6. A suggested pathway of evolution of the albumin superfamily, derived in large measure from the schemes of Gray and Doolittle (1992) and Haefliger *et al.* (1989). Suggested times are highly approximate. The scheme proposes that albumin evolved from a one-domain precursor, perhaps in a subvertebrate stage; lamprey albumin diverged before the three-domain albumin appeared; DBP is older than the albumins of species other than lamprey; ALF and then AFP diverged from albumin and DBP after the bony fishes appeared; loop 1 was originally a closed loop; and albumin lost its carbohydrate between the teleost and reptilian radiations. The hagfish is now proposed to be more ancient than the lamprey and to have evolved independently (Forey and Janvier, 1993). My, Million years.

truncation to 2.5 domains. The three-domain albumin persists thereafter, containing 9 intact double loops and carrying carbohydrate. At about the time of the amphibian radiation (400 My), ALF and then AFP split off (400–300 My), losing the Cys 53–43 pair as well as (in AFP) the Cys 316–361 pair forming loop 6; half-Cys-8 moved to CySH-13. In the amphibian albumins and above, Cys-53 in loop 1 was likewise lost, but half-Cys-8 moved to CySH-34; loop 6 remained intact. AFP changed even more rapidly than did HSA, 24% compared to 18%/100My; DBP evolved with time at about 15%/100My (Haefliger *et al.*, 1989).

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In some amphibian alleles and in reptiles, birds, and mammals, albumin no longer contains carbohydrate. Tryptophan disappears in albumin of birds. Within the mammals, there is a single tryptophan except in artiodactyla; residue 116 is absent in hoofed animals.

The reptilians, the first wholly terrestrial animals, appear to have been more radical in their modifications of albumin. During their long history, not only has cobra albumin developed its own S–S loop structure but an ancient *Sphenodon*, the tuatara of New Zealand, has been found to have albumins of 120 and 130 kDa (Brown, 1993).

Major questions remain. The point of the lamprey separation is not clear; this cartilaginous, jawless animal with a life cycle of profound metabolic changes is actually highly evolved rather than primitive. The report of a protein with properties of AFP in sharks (Gitlin *et al.*, 1973) does not fit with its absence in teleosts; is this yet another ancestral member of the superfamily? Above all, the albumin anlage is elusive. Sequence homologies predict that the original albumin had a single domain, about 190 amino acids, and before that may have evolved from a single loop of about 65 residues. This loop (loop 7, coded by exons AB of domain III?) could have mutated by gene duplication to yield exons ABCD; partial duplication followed by partial excision yielded domain III (Eiferman *et al.*, 1981). Ohno (1981) predicted that the original domain arose from repeated sequences, particularly FTEEQL and FMEE (in one-letter code) found today in bovine albumin and AFP. These sequences do not, however, persist in albumins of older species.

Where would one look for this anlage? The hagfish lineage appears to be even more ancient than that of the lamprey (Forey and Janvier, 1993); might the albumin of this cyclostome have a different number of domains than does the lamprey—or might it even be the sought-after single-domain albumin?

Below the chordates the evolutionary trail has a large gap, blazed only by suggestions that vertebrates evolved from the larval stage of tunicates (Romer, 1967) or from free-living cephalochordates (Wada and Satoh, 1994). But no 190-residue fatty acid-binding protein is as yet apparent among invertebrates. The lipophilins and other fat-transport proteins of insects appear not to be albumin relatives. Searching of circulatory fluids of 15 invertebrate species, including *Amphioxus*, by the author, failed to discern any palmitate-binding protein in this size range.

In the case of another plasma protein, transferrin, however, a half-size, one-domain, version has been found in echinoderms (Martin *et al.*, 1984). Other plasma proteins can also be traced beyond the chordate cutoff; a fibrinogen-like sequence appears in the sea cucumber, an echinoderm (Xu and Doolittle, 1990), and a homolog of α_2-macroglobulin occurs in the hemolymph of the horseshoe crab, an arachnid (Quigley and Armstrong, 1985). As Neurath has noted (1986), lending of domain structures among proteins may have been a shortcut of early

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evolution, such "exon shuffling" being made easier by the frequent occurrence of introns.

IV. MUTANT FORMS

In 1955 Scheurlen recognized a double albumin band in the electrophoretic pattern from a 25-year-old woman with diabetic ketosis. The abnormal (slower) band disappeared in a few months after her condition had improved. The report was little noticed until Wuhrmann (1959) found that several of the patient's close relatives—father, brother, and son—also showed a second albumin, but one that was not transient.

Meanwhile Knedel (1957) and Nennstiel and Becht (1957) had also reported slower albumin bands on electrophoresis, each band amounting to half of the normal albumin concentration. Knedel found the abnormal band to be enduring, and to be present in five of seven of the subject's family. These were cases of heterozygotic alloalbuminemia. The transient types result from alteration of mobility by a bound drug or other ligand as above (Chapter 3, Section I.C.3) or by proteolytic cleavage incident to pancreatitis, or from leakage of proalbumin from the liver cell (Chapter 6, Section II.B.4.a). In the transient bisalbuminemias the abnormal band is usually much weaker than that of the normal albumin, whereas in the hereditary form both bands are usually of equal concentration.

The prevalence of albumin alleles detected by electrophoresis in the average human population is low, 0.0003–0.001, too low to be termed polymorphism, which generally means prevalence greater than 0.01. Only about one-third of amino acids are charged, however, so that about half of single-residue substitutions would not alter the net charge of the albumin and would probably be undetectable by electrophoresis. Only one variant has been detected by an alteration of function, the albumin form with increased affinity for thyroxine in familial dysalbuminemic hyperthyroxinemia (FDH). Useful reviews on mutant forms of albumin are those by Blumberg *et al.* (1968), Tárnoky (1980), Arai *et al.* (1990), and Porta *et al.* (1992).

A. Classification

In 1958 Earle *et al.* found a slow albumin band, which they termed Albumin B, in the serum of 25 of 56 members of an American family of Norwegian heritage. Tárnoky and Lestas (1964) found the fast-moving Albumin Reading in England, and established the practice of naming a variant for the geographical area of its discovery. Albumin Naskapi was next discovered by Melartin and Blumberg (1966) during a survey of gene frequencies in the North American Indians and Eskimos. This study established hereditary bisalbuminemia as the

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result of expression of both alleles of the single albumin gene. Penetrance is complete because half of the total albumin is contributed by each allele; there is no suppression by the presence of a mutant gene.

Since the mid-1960s the number of reported allotypes has exceeded 100. Clinical electrophoresis on cellulose acetate at pH 8.6 gave sharper separations than on filter paper, but identification by the position of a band was still difficult. Spurred by collection efforts of F. Porta in Sondrio, Italy (Porta *et al.*, 1979), J.M. Fine in Paris (Fine *et al.*, 1982), and L. Weitkamp in Rochester, New York (Weitkamp *et al.*, 1973), a classification system was derived, using migration at three pH values—5.0, 6.9, and 8.6—and about 50 mutants have been classified. The "CISMEL" group (Comitato Italiano di Standardizzazione in Medicina di Laboratorio) (Burlina *et al.*, 1985) proposed that classification be based on electrophoretic migration relative to that of human transferrin as a reference point, but that ultimately they should be classified by the amino acid substitution (and, we would hope, eventually by the DNA base substitution). Naming is for the geographical origin, not the laboratory where first detected or the family name of the proband. This rule is now generally followed except for well-established names such as Indian tribes—Naskapi, Yanomama, etc. Normal albumin is termed Albumin A, but should not be confused with the A (aged) isomeric form (Chapter 2, Section II,C,1,d).

Identification of eight variant forms by electrophoresis at pH 8.6 is illustrated in Fig. 4-7. All of these are of the slow type relative to Albumin A. Allotypes that overlap at pH 8.6, such as Christchurch (-1 Arg $\rightarrow$ Gln, lane 3, Fig. 4-7) and Lille (-2 Arg $\rightarrow$ His, lane 9, Fig. 4-7) will separate at pH 5 because the histidine ionizes in the pH 6 range. Isoelectric focusing has recently been applied as a second stage of classification of an albumin variant on the basis of its isoelectric point (Rochu *et al.*, 1991). The heterogeneity of the usual albumin preparation in this technique can be a source of confusion, however.

B. Molecular Location of Mutations

A substitution of Lys for Glu was detected in comparing a peptide from digests of Albumins A and B in 1961 (Gitlin *et al.*, 1961), and was positioned at residue 570 when the complete sequence of HSA became available in 1975. With major efforts by workers such as F.W. Putnam, S.O. Brennan, and M. Galliano, the locations of a total of 53 such mutations have now been identified (Table 4-8). Putman and co-workers developed a system of tandem HPLC separation of a proteolytic digest, which speeded the work by allowing facile detection of abnormal peptides (Takahashi *et al.*, 1986). Increasingly the site of mutation in the albumin gene is being identified.

The number of known variants is greater for albumin than for any other protein except hemoglobin (Arai *et al.*, 1990). For at least 16 mutations the DNA

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Fig. 4-7. Electrophoretic detection at pH 8.6 of some mutant HSA forms from Table 4-8. Specimens are plasma diluted 1:10 and contain $^{63}Ni^{2+}$. N, Normal albumin; lane 1, −2 Arg → Cys; lane 2, same, homozygous; lane 3, −1 Arg → Gln; lane 4, 268 Gln → Arg; lane 5, 318 Asn → Lys; lane 6, 376 Glu → Gln; lane 7, 550 Asp → Ala; lane 8, 570 Glu → Lys; lane 9, −1 Arg → His. (A) Coomassie Blue stain. (B) Autoradiograph of same gel, showing forms binding Ni^{2+}; the proalbumin forms in lanes 1, 2, 3, and 9 do not bind nickel because the α-amino nitrogen of Asp-1 is blocked. Note that the mutant form is equal to the normal A form in concentration form except for −2 Arg → Cys, which has defective signal peptide cleavage. Reproduced from Carlson *et al.* (1992) by permission of the National Academy of Science.

base change has been determined. As seen in Table 4-8, mutants occur in three types: single-base substitutions, accounting for most of the variants; frameshifts (#567 and #580), causing substitutions such as -KLP for the C-terminal sequence QAALGL; and intronic mutations causing splicing errors. In two of the latter, the usual C-terminal sequence beginning at 572, GKKLVAASQAALGL, from exon 14, is missing. In its place is the continued translation of either intron 13, LLQF-SSF (Albumin Rugby Park), or of exon 15 rather than exon 14, PTMRIRERK (Albumin Venezia), until a terminator codon is encountered. The C terminus of Albumin Venezia is "ragged," lacking either or both of the terminal RK pair, owing to the action of carboxypeptidase B in the plasma.

Five of the mutations occur in the propeptide of proalbumin; the first of these was detected by Brennan and Carrell (1978). Four are pictured in Fig. 4-7, lanes 1–3 and 9. Each modifies the terminal Arg-Arg sequence that defines the cleavage site, so that the normally intracellular proalbumin (Chapter 5, Section I,D) is secreted without cleavage of its propeptide. Hypermutability of CpG dinucleotides in the codons for these two positions has been proposed to account for the prevalence of alterations at this region (Brennan *et al.*, 1990a). One of them, Albumin Malmö I (Redhill), carries an unusual double mutation, −2 Arg → Cys and 320 Ala → Thr.

The distribution of mutant sites in the albumin molecule is depicted in Fig. 4-8. It is decidedly nonrandom. Rather, most mutations occur at the amino terminus, including those in the propeptide, and in subdomains IIB and IIIB, residues 313–382 and 479–580 around loops 6 and 9, respectively. Except for 218 Arg → His, these residues avoid the major ligand sites in subdomains IIA and IIIA (Fig. 2-9), and also avoid the stretches of peptide chain that are highly conserved among different species (Fig. 4-3). This dispersion is in accord with the benign nature of most albumin mutants; some appear to have minor effects

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on ligand binding (see Section IV,D), but none is known to cause disease. All but 218 Arg → His and 320 Ala → Thr appear to be on the surface of the albumin molecule where they do not cause significant structural changes.

C. Distribution in Populations

1. Distribution in Humans

In Europe the predominant variant alleles are Albumin Lille (−2 Arg → His), Proalbumin Christchurch (−1 Arg → Gln), and Albumin B (570 Glu → Lys) (Arai *et al.*, 1990) at a total frequency of heterozygotes of 0.003. A survey of nearly 200,000 serum samples in Sweden found Albumin Lille plus Albumin Tagliacozzo (313 Lys → Asn), with a frequency of 0.006 (Carlson *et al.*, 1992). Porta kept careful records of the distribution of nine allotypes in Italy; his charts (Burlina *et al.*, 1985; Porta *et al.*, 1992) show the predicted dominance of variants in a local area: Albumin Milano in Lombardia; Albumins Milano, Verona, and Venezia in Veneto; and Albumin Catania in Sicily. The latter is the most restricted geographically, in accord with the social seclusiveness of Sicilians. Three homozygotic subjects have been found in Italy and Sweden, the result of intermarriage of parents both carrying Albumin Lille. Detection of homozygotes is difficult, because there is no bisalbuminemia and the only clue is a small change in electrophoretic migration rate. Note the homozygous pattern in lane 2 of Fig. 4-7.

Asian surveys, including 16,000 specimens from Hiroshima and Nagasaki assessing radiation-induced changes, found two variants from Hiroshima and three from Nagasaki, too few to place the blame on excessive radiation, with a total of 13 alleles in Japan and an incidence of 0.0016; of these 13, 10 are unique to that country (Neel *et al.*, 1988). Other Asian forms are Taipei (= Lille), New Guinea (= Tagliacozzo), Mersin (= Naskapi), Lambadi (= Maneus I), and Phnom Phen (= B) (Arai *et al.*, 1990). Only one variant from Africa has been detected, Albumin Lille in a Somalian living in Italy.

The polymorphic occurrence of Albumin Naskapi (372 Lys → Glu) has helped to document the migration of Indian tribes in North America. In the Algonquian-speaking Naskapi of Labrador prevalence was 0.138, and 4 homozygotes were noted in 93 subjects. In the Athabaskan-speaking Indians of Alberta the frequency was 0.022. The allele extends southward in the United States to the Athabaskan-speaking Navajos and Apaches (occurrence 0.016) of the desert Southwest, where it is found mixed with Albumin Mexico-2 (550 Asp → Gly), frequency 0.037 in Apaches. The interpretation is that ancestral Americans migrated across the Bering Strait 15,000–20,000 BC and then branched eastward to Canada and southward

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TABLE 4-8
Locations of Mutations of Human Albumin[a]

Residue	Amino acid change From	To	Codon change[c]	Geographical names and references; in order of reports[b]
−2	Arg	His	G<u>C</u>T C<u>A</u>T +	Lille[1]; Pollibauer, Somalia, Tokushima,[2] Taipei,[3] Fukuoka-2,[4] Varese[5]; Wu Yang[6]; Mayo, Komagone-3[6,7]
2[d]	Arg	Cys	<u>C</u>GT <u>T</u>GT +	(−2 to −6 omitted) Malmö-I,[8] Kaikoura,[9] Tradate,[5] Redhill (+320)[10,11]; high in Italy, Sweden, 3 homozygotes[11,12]
1	Arg	Gln	C<u>G</u>A C<u>A</u>A +	Christchurch[13]; Gainesville,[3,14] Y, Honolulu-2,[4] Fukuoka-3[4]; Mayo[6,7]; Shizuoka[15]
−1	Arg	Pro	C<u>G</u>A C<u>C</u>A	Takefu[3]; Honolulu-1[3]
1	Arg	Leu	C<u>G</u>A C<u>T</u>A	Jaffna[16]
1[e]	Asp	Val	G<u>A</u>T G<u>T</u>T	Blenheim[17]; Bremen[18]; Malmö-II; Iowa City-2[7]
3	His	Gln	CA<u>C</u> CA<u>A/G</u>	Nagasaki-3[19]
3[e]	His	Tyr	CA<u>C</u> TAC +	Larino,[20]
32	Gln	Stop	<u>C</u>AG TAG +	Analbuminemia case 18[21]
60	Glu	Lys	G<u>A</u>A <u>A</u>AA	Torino[5]
63	Asp	Asn	G<u>A</u>C <u>A</u>AC CHO	Dalakarlia, SW-1; CHO next to Cys[12]
82	Glu	Lys	G<u>A</u>A <u>A</u>AA	Vibo Valentia[5]
114	Arg	Gly	C<u>G</u>A G<u>G</u>A	Yanomama-2[19]
114	Arg	Stop	C<u>G</u>A T<u>G</u>A +	Analbuminemia case 3[21]
119	Glu	Lys	G<u>A</u>G <u>A</u>AG	Nagoya[18]
128	His	Arg	C<u>A</u>T C<u>G</u>T	Komagome-2[7]
140[d]	Tyr	Cys	T<u>A</u>T T<u>G</u>T	Asola[22]
177[d]	Cys	Phe	T<u>G</u>C T<u>T</u>C	Hawkes Bay[23]
214	Intron 6, 3′		<u>A</u>G G<u>G</u> +	Analbuminemia case 16[24]
214	Intron 6, 3′		G/<u>G</u> G/<u>A</u> +	Analbuminemia case 15[21] (Trp[214] → stop)
218	Arg	His	C<u>G</u>C C<u>A</u>C +	Familial dysalbuminemic hyperthyroxinemia (FDH)[25,26]
225	Lys	Gln	<u>A</u>AA C<u>A</u>A +	Tradate-2[4,20]
240	Lys	Glu	<u>A</u>AA G<u>A</u>A	Herborn[27]
267	Exon 8		<u>A</u> insertion +	Analbuminemia, cases 10, 11[28]
268	Gln	Arg	C<u>A</u>A C<u>G</u>A	Skåne SW12

(continues)

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TABLE 4-8—*Continued*

Residue	Amino acid change From	To	Codon changes[a]	Geographical names and references; in order of reports[b]
269	Asp	Gly	GAT GGT	Niigata,[29] Nagasaki-1[15]
276	Lys	Asn	AAG AAC	Caserta[20]
313	Lys	Asn	AAG AAT +	Tagliacozzo[30]; Cooperstown[31]; Canterbury,[32] New Guinea[18] (Reading?[33]), IRE-1[34a,12, 20]
318	Asn	Lys	AAG AATAC	Örebro SW, Malmö-4[12]
320	Ala	Thr	GCT ACT CHO	Redhill[11, 35] (gives Asn-Tyr-Thr site for glycosylation); also - 2 Arg → Cys (Malmö-1)
321	Glu	Lys	GAG AAG	Roma[36]
333	Glu	Lys	GAA AAA	Sondrio[37, 38]
354	Glu	Lys	GAA AAA	Hiroshima-1[15]
358	Glu	Lys	GAG AAG	Coari-1, Porto Alegre-1[39]
365	Asp	His	GAT CAT	Parklands[40]
365	Asp	Val	GAT GGT	Iowa City-1[7]
372	Lys	Glu	AAA GAA	Naskapi,[41] Mersin[42]; Komagone-1[7]
375	Asp	Asn	GAT AAT	Nagasaki-2[19]
376	Glu	Lys	GAA AAA	Tochigi[15]
376	Glu	Gln	GAA CAA	Malmö-3[12]
382	Glu	Lys	GAA AAA	Hiroshima-2[15]
479	Glu	Lys	GAA AAA	Dublin[34]
494	Asp	Asn	GAT AAT CHO	Casebrook[43]
501	Glu	Lys	GAG AAG +	Vancouver, Brimingham, Adana[44]; Porto Alegre-II,[39] Manaus-I,[39] Lambadi, c.[18] Kashmir[45, 46]
505	Glu	Lys	GAA AAA	Ortonovo[47]
536	Lys	Glu	AAG GAG	Castel di Sangro[48]
541	Lys	Glu	AAA GAA	Makú (Wapishana)[39]; Oriximina-1[39]
550	Asp	Gly	GAT GGT	Mexico[42]
550	Asp	Ala	GAT GCT	Dalakarlia, Malmö-62[12]
563	Asp	Asn	GAT AAT+	Fukuoka-1[18]; Ube-1; Varese-2, Paris-2[38,20]
565	Glu	Lys	GAG AAG	Osaka-1[18]

(continues)

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TABLE 4-8—*Continued*

Residue	Amino acid change From	To	Codon changes	Geographical names and references; in order of reports[b]
567[c]	Cys	—	TGC _GC +	Bazzano[9,20] (frameshift: CFAEEGKKL-VAAASQAALGL → ALPRRVKNLLQVKLP)
570	Glu	Lys	GAG AAG +	B, Oliphant[49]; Verona[9,20,50]; Osaka-2[4,15]; Phnom Penh[4]; Nagano; Iowa City-3; Mayo[7]; Victoria (East India); Saitama-1[15]
572[f]	Intron 13-5′		G C	Rugby Park[51] (exon 14 omitted: GKKLVAASQAALGL → LLQFSSF)
572[f]	Exon 14-5′		GT TT	Venezia[52,53] [exon 14 omitted: GKKLVAASQAALGL → PTMRIRE(R)(K)]
573	Lys	Glu	AAA GAA +	Milano fast[20,54]
574	Lys	Asn	AAA AA T/C	Vanves[50]
580	Gln	—	CAA _AA	Catania[53,55] (frameshift: QAALGL → KLP)

[a]As of October, 1994.

[b]Malmö-I, 3% proalbumin, 30% Arg-albumin, due to aberrant signal peptide cleavage.

[c]Blenheim, 10% proalbumin, 40% Val-; Bremen, 20% Arg-Alb, 30% Val-; Larino, 10–12% variant.

[d]Asola, 25–45% variant; Hawkes Bay, 5% variant; Bazzano, 18% variant.

[e]Caserta, 60–70% variant.

[f]Rugby Park, 8% variant; Venezia, 30% variant.

[g]+, Indicates a known change.

[h]Key to references: (1) Abdo *et al.* (1981), (2) Matsuda *et al.* (1986), (3) Takahashi *et al.* (1987c), (4) Arai *et al.* (1989b), (5) Galliano *et al.* (1990), (6) Zan *et al.* (1993), (7) Madison *et al.* (1991), (8) Rousseaux *et al.* (1982), (9) Brennan *et al.* (1990a), (10) Brand *et al.* (1984), (11) Hutchinson and Matejtschuk (1985), (12) Carlson *et al.* (1992), (13) Brennan and Carrell (1978), (14) Fine *et al.* (1983), (15) Arai *et al.* (1989c), (16) Galliano *et al.* (1989), (17) Brennan *et al.* (1989), (18) Arai *et al.* (1990), (19) Takahashi *et al.* (1987b), (20) Madison *et al.* (1994), (21) Watkins *et al.* (1994b), (22) Minchiotti *et al.* (1995), (23) Brennan and Fellowes (1993), (24) Ruffner and Dugaiczyk (1988), (25) Petersen *et al.* (1994), (26) Sunthornthepvarakul *et al.* (1994), (27) Minchiotti *et al.* (1993), (28) Watkins *et al.* (1994a), (29) Sugita *et al.* (1987), (30) Galliano *et al.* (1986a), (31) Huss *et al.* (1988b), (32) Brennan and Herbert (1987), (33) Tárnoky and Lestas (1964), (34) Sakamoto *et al.* (1991), (35) Brennan *et al.* (1990b), (36) Galliano *et al.* (1988), (37) Porta *et al.* (1992), (38) Minchiotti *et al.* (1992), (39) Arai *et al.* (1989a), (40) Brennan (1985), (41) Franklin *et al.* (1980), (42) Takahashi *et al.* (1987a), (43) Peach and Brennan (1991), (44) Huss *et al.* (1988a), (45) Savva *et al.* (1990), (46) Tárnoky *et al.* (1992), (47) Galliano *et al.* (1993), (48) Minchiotti *et al.* (1990),(49) Winter *et al.* (1972), (50) Minchiotti *et al.* (1987), (51) Peach *et al.* (1992), (52) Minchiotti *et al.* (1989), (53) Watkins *et al.* (1991), (54) Iadarola *et al.* (1985), (55) Galliano *et al.* (1986b).

[i]Reference reporting nucleotide base change.

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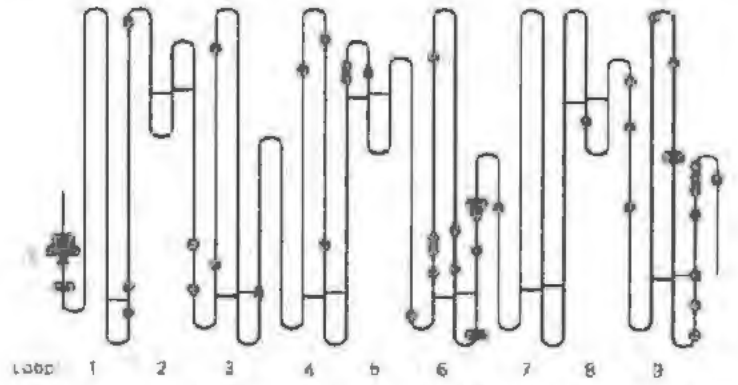


Fig. 4-8. Schematic locations on the human proalbumin molecule of mutations that generate viable circulating forms (Table 4-8).

toward Mexico (Schell *et al.*, 1978). Albumin Naskapi was also known as Albumin Mersin among Eti Turks, and is found in the Punjab region of India (Kaur *et al.*, 1982); here it represents either a common Asian ancestor to the Indian tribes or chance independent mutations (Takahashi *et al.*, 1987a).

Studies in South America have also helped to define the relationship among tribes such as the stone-age Brazilian Yanomama (frequency 0.08). The Makú and Oriximina I, and Coari I and Porto Alegre I, were found to be the same mutations (Arai *et al.*, 1989a), restricted to South America, whereas the Manaus-I and Porto Alegre-II changes occur at the same residue as albumins from Vancouver, Birmingham, Adana, and Lambadi and Kashmir in India. This also appears to be the result of independent mutations.

Another possible instance of independent mutation at widely separated geographical sites is Albumin Tagliacozzo (Italy) (313 Lys → Asn), which has been found in Canterbury (New Zealand), Cooperstown (New York), Ireland, Sweden, and with high frequency in New Guinea (Carlson *et al.*, 1992). Albumin Lille (France) (−2 Arg → His), also in Japan, Taipei, Varese (Italy), and the United States, is another candidate for independent mutation.

Over 100 alleles of the close albumin relative, DBP, have been identified (Kamboh and Ferrell, 1986). Haplotype frequencies of albumin, DBP, and hemoglobin have been interpreted to indicate that the Micronesians and Polynesians derived from Southeast Asia, whereas Melanesian populations originated independently.

2. *Distribution in Other Animals*

Albumin polymorphism appears to be more common in subprimate species than in humans and has been reported in every class of animals. Five

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bands were observed in carp (Lukianenko *et al.*, 1971), and two in rainbow trout, which is tetraploid with two albumin genes (Davidson *et al.*, 1989). The polymorphism in the amphibian *Xenopus* was described in Section III.A.3; one form is glycosylated and the other is not. Other amphibian examples are the hylid frogs (Dessauer *et al.*, 1977), the newt (Francis *et al.*, 1985), and the toad, in which 29 phenotypes of 11 albumin alleles were observed (Guttman and Wilson, 1973).

In reptiles, polymorphism has been noted in the turtle (Lykakis, 1971) and the king snake, the latter having a fast allotype in the Eastern United States, a slow one in the West, and a hybrid form in western Texas (Dessauer and Pough, 1975). The chicken (Jernigan *et al.*, 1973) and quail (Haley, 1965) are examples of birds with polymorphic albumins.

Mammalian species showing multiple albumin alleles include the rabbit (Ferrand and Rocha, 1992), the Equidae (horse, donkey, mule, zebra) (Osterhoff, 1966), cattle (Soos, 1971; Panepucci and Vicente, 1991), water buffalo (Tan *et al.*, 1993), and sheep (Tucker, 1968). The domestic pig, *Sus scrofa*, is of interest in having three alleles, O, A, and B (Kristjansson, 1966). The O is a null allele, producing no albumins. Hence the OA and OB hybrids have only half the albumin level of the AB form, and the OO heterozygote would be analbuminemic.

D. Effects of Mutations on Albumin Molecule

The variation in electrophoretic mobility is often, but not always, predictable from the charge change of the mutant: note that mutant forms in lanes 4–7 of Fig. 4–7 have a single + charge addition, yet migrate somewhat differently; the +2 charge change in lane 8 shows much better separation. Conformational factors may be significant. The 333 Glu → Lys Albumin Sondrio migrates together with Albumin Paris-2, 563 Asp → Asn (Minchiotti *et al.*, 1992). These authors propose that the +1 charge gain in domain III has as much effect as the +2 gain in domain II because of greater exposure of the pertinent residues in domain III. Another factor that can affect the migration rate is glycosylation with the accompanying sialyation (see below).

Three variants to date carry mutations that produce an N-glycosylation site. Each is utilized to some extent, causing an increase in molecular mass of about 2.5 kDa from the carbohydrate chain addition. In Albumin Casebrook the 494 Asp → Asn change creates an Asn-Glu-Thr acceptor signal that is reported to be fully utilized to attach a biantennary carbohydrate chain, but with degradation of about 15% of the glycosylated albumin in the circulation. In Albumin Dalakarlia, 63 Asp → Asn, an Asn-Lys-Ser site is created adjacent to the disulfide bond involving Cys-62; only about half of the variant albumin is found to

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be glycosylated, perhaps a result of steric hindrance. On the other hand, glycosylation at the second mutant position of Albumin Redhill, 320 Ala → Thr, appears complete even though its Asn-Tyr-Thr site is separated from a disulfide bond by only one residue.

The molecular mass of mutants is usually not materially changed. Exceptions would be glycosylated forms and those that are truncated (Rugby Park, Venezia, Catania) or carry a variant propeptide or remnant of one at the amino terminus. Nor is the turnover of a variant form in the circulation altered (Mariani *et al.*, 1978) except in the presence of structural alterations as described below.

The variant forms at the amino terminus are of special interest. Changing the terminal Arg-Arg sequence of the propeptide blocks its normal intracellular cleavage, but may create a new site for signal peptide cleavage (Chapter 5, Section I.C) (Brennan *et al.*, 1990b). Thus Albumin Redhill, −2 Arg → Cys, appears in the plasma as 3% proalbumin and 30% Arg-albumin, with the Arg-Gly-Val-Phe-Cys pentapeptide removed (Fig. 4-7). The remaining ≈17% probably was converted to Albumin A on removal of the terminal Arg by circulating serine proteases. In Albumin Blenheim, 1 Asp → Val, propeptide cleavage is faulty and about 10% appears in the plasma as the proalbumin form carrying the full propeptide and about 20% carrying amino-terminal arginine.

Albumin Christchurch (−1 Arg → Gln) is unstable and disappears (converts to Albumin A) on storage of plasma (Rousseaux *et al.*, 1982). The "disappearance" was attributed to cleavage at the −2 Arg-Gln site by plasmin, leaving Gln-albumin, which would not be readily detectable by electrophoresis because its charge is unaffected. The aberrant propeptide forms with substitutions at residues −2 or −1 have been useful in delineating the specifity of proposed intracellular proalbumin processing enzymes (Brennan *et al.*, 1989).

Another substitution affecting a cleavage is 365 Asp → His (Albumin Parklands) in which the sole Asp-Pro site in HSA becomes His-Pro. This causes Albumin Parklands to be more stable than Albumin A to acid conditions, which can cause cleavage at the Asp-Pro site (Brennan, 1985).

Three mutations appear to affect disulfide bonding. The 177 Cys → Phe substitution in Albumin Hawkes Bay obviates the S–S bond between Cys-168 and Cys-177. Brennan and Fellowes (1993) conjectured that Cys-168 might bind instead to nearby Cys-124, causing a gross conformational change. Such a change is implied by the slow migration of Albumin Hawkes Bay on agarose gel electrophoresis, even though the calculated difference is zero, with the migration reverting to that of Albumin A in the presence of 5 m*M* dithiothreitol. The conformational change also causes the molecule to be unstable and readily catabolized, so that only 5% of the variant form persists in plasma (Table 4-8).

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The 140 Tyr → Cys mutation in Albumin Asola is proposed to create an eighteenth S–S bond in this protein, between the new Cys and CySH-34. Albumin Asola shows no free SH, but has an apparent lower Stokes radius on SDS-gel electrophoresis that reverts to the normal range under reducing conditions. In two subjects the variant form constituted 25 and 45% of the total albumin. In Albumin Bazzano Cys-567 is absent, so the final S–S bond cannot be formed; only 18% of the variant form circulates.

Ligand binding has rarely been shown to be affected in variant albumins. Electrophoretic observations on serum after adding a candidate ligand are confused by shifts of a ligand from one albumin band to the other during migration. The effort of obtaining pure preparations of both a variant and Albumin A from the same subject has discouraged comparisons of isolated proteins, so that some reports have compared drug binding by a variant to that by commercially prepared Albumin A. Effects appear to be largely nonspecific or perhaps the result of configurational differences (see Chapter 3, Sections I,C,4 and I,D).

One ligand report that seems free of such complications shows decreased total bilirubin binding capacity by Brazilian Indians homozygotic for Albumin Yanomama-2 compared to those homozygotic for Albumin A (Lorey *et al.*, 1984). But heterozygotes showed the full capacity of Albumin A when they should show only an intermediate capacity, and the Yanomama Indians do not appear to suffer from poor transport of bilirubin. The site of the substitution, 114 Arg → Gly, is somewhat remote from the suspected bilirubin binding site on albumin in the region of residues 195–251 (Chapter 3, Section I,B,3). If the effect is real, the charge of the inserted arginine may have altered a critical aspect of the protein structure to cause a more global effect. The mutation in the macaque, 188 Glu → Gln, is somewhat closer and causes a modest depression of bilirubin binding (Watkins *et al.*, 1993).

An obvious defect in ligand binding is the predicted failure to bind copper(II) or nickel(II) by mutants in which the amino-terminal site, Asp-Ala-His (Chapter 3, Section II,A,1), is altered. This includes all of the proalbumins (Table 4-8), in which the persistent propeptide blocks the α-NH_2 of the normal terminal residue, and Albumin Nagasaki-3, 3 His → Gln, in which the requisite imidazole at residue 3 is missing. A failure to bind Ni/Cu is therefore a characteristic of such variants, and has been introduced as a simple test for their detection; the weak beta emitter, ^{63}Ni, can be detected by autoradiography after addition to a sample before electrophoresis (Peters and Reed, 1980), as demonstrated in Fig. 4-7, or, avoiding the use of radioactive techniques, by colorimetric detection of bound copper(II) (Rochu and Fine, 1986). A confirmatory test for proalbumin is reversion of its migration to that of Albumin A after mild treatment with trypsin (≈2 μg/mL, pH 8.6), which cleaves off the propeptide or single arginine residue from the amino terminus.

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The albumin variant in FDH (Chapter 3, Section I,D,2) that appears to bind thyroxine with abnormally high affinity has recently been identified. The mutation 218 Arg → His was found in a total of 10 unrelated heterozygotes. The change creates a new binding site for thyroxine in Site I (subdomain IIA) in addition to the purported one in subdomain IIIA. *In vitro* tests of thyroxine binding with whole plasma showed the $\bar{v}$ of the strong thyroxine site to be 0.5 rather than 1.0 (Barlow *et al.*, 1986). Concurrent presence of the RFLP *Sac*I+ (CTC) sequence at residue 532 in exon 13 in every individual with FDH strongly suggested a founder effect of remote common ancestry.

Only six mutation sites coincide with purported antigenic determinants (Chapter 3, Section III), and for only one mutant has an immunological difference been reported. This variant was discovered in London, Ontario (Naylor *et al.*, 1982), and was subsequently shown to be in Albumin B (Arai *et al.*, 1989b). The mutation in Albumin B, 570 Glu → Lys, lies adjacent to a purposed antigenic locus of HSA, residues 561–567 + 555–558 (Chapter 3, Section III,B).

Because the known mutations nearly all occur in superficial, nonfunctional sites, can we presume that amino acid substitutions in critical internal areas produce such aberrant structure and the loss of such crucial ligand handling for the fetus that they are lethal *in utero* even when heterozygous?

E. Genetics of Analbuminemia

In 1954 Bennhold *et al.* at Tübingen were amazed to find no detectable albumin in the electrophoretic pattern of a 31-year-old daughter of a farmer. She had been referred because of an elevated erythrocyte sedimentation rate, premenstrual ankle edema, and fatigue, but otherwise appeared healthy and could do a full day's work in the fields. This was the first case of analbuminemia, the apparent total absence of albumin from the blood.

1. Definition: The "Analbuminemia Register"

By 1985, 28 cases had been found in 24 families. Because survival without albumin cast doubt on the need for this most abundant protein of the blood plasma, R.G. Reed and the author started an "Analbuminemia Register," with the purpose of monitoring all known and future cases to learn the consequences of life without albumin, and thus perhaps gain an idea of its primary function. The current "Register" is summarized in Table 4-9. The genetic basis of analbuminemia is discussed in this section, whereas the clinical aspects of this condition are discussed in Chapter 5 (Section II,C), with regard to the function of albumin. Suffice it to note here that the average age at detection is 24 years

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(Table 4-9), that the main functional sign is some degree of edema and fatigue, with hyperlipidemia a common finding but without resultant atherosclerosis, and that even longevity is little unaffected. Only 6 of the 28 subjects are believed to have died, at an average age of 59 years.

Electrophoretic measurements of albumin concentration in serum are not accurate in the lower range (below 5 g/L) and when immunochemical assay methods were applied very small amounts of albumin were invariably detected (Table 4-9). Discounting the electrophoretic assays, amounts ranged from 16 to 1200 mg/L, with a mean of 23 mg/L. Thus, the condition is not truly analbuminemia. Some of the higher values may have been influenced by administration of intravenous albumin before the nature of the condition was realized. Some others, case 20 at Ann Arbor, for instance, had persistent values near 10 g/L and may actually be hypoalbuminemia. An arbitrary upper limit of 1 g/L in untreated subjects has been proposed for classifying future cases as analbuminemia.

2. *Hereditary Features*

The brother of the index case was soon found also to lack albumin (case 2, Table 4-9). An extensive genealogic survey showed that both parents of these siblings had subnormal albumin levels, ≈32 g/L, and that intermarriage had been common; in fact, at the fourth ancestral generation there were only 10 great-great-grandparents instead of the usual number of 16!

In 15 of the 28 known cases consanguinity has been shown to be a factor. Cases 10 + 11 and 12 + 13 + 14 were familial; three of five children in the latter family were affected. Lineage studies of cases 9 and 16 supported the concept that analbuminemia is an inherited genetic condition resulting from mutation in one of the codominant albumin alleles. The homozygous condition produces little or no albumin; in the heterozygous state the single normal allele is sufficient to produce more than half the normal amount of albumin in the absence of a functional partner. The parents of case 16 showed 33 and 28 g/L (Boman *et al.*, 1976) and the parents and one sibling of cases 10 and 11 had circulating albumin levels of 46–49 g/L (Watkins *et al.*, 1994a).

Further proof of the mode of inheritance of analbuminemia was the establishment of a strain of analbuminemic rats. Termed the Nagase strain or "NAR" (Nagase analbuminemic rat), it was derived from an analbuminemic Sprague–Dawley rat found in 1977 in Tokyo (Nagase *et al.*, 1979). Inheritance was autosomal recessive. As with humans, a minute amount of circulating albumin (5 mg/L) is still detectable. This strain has been maintained and has been the basis for many useful studies (Nagase, 1987) (Chapter 5, Section II,C).

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Albumin degradation is not excessive in either analbuminemic man or rats. In fact, labeled albumin disappears abnormally slowly after injection. Hence the primary defect in analbuminemia is one of albumin synthesis, a defect readily demonstrated in rats; no functional mRNA is found in their liver cells (Esumi *et al.*, 1980).

3. Genetic Basis

In both analbuminemic humans and rodents, as determined by RFLPs, the gene for albumin is still present (Murray *et al.*, 1983). Two types of mutation have thus far been identified, mRNA splicing errors and generation of premature stop codons that terminate translation prematurely.

A splicing error was found in the Nagase rat (Esumi *et al.*, 1983), and a few years later in an analbuminemic human. In the rat, a 7-bp deletion occurs in intron HI (corresponding to human intron 8), extending from bp 5 to 11 from its 5' end. The deletion can be observed in the nuclear mRNA transcribed from the analbuminemic gene as well, where it causes splicing errors on intron removal (Shalaby and Shafritz, 1990), and apparently leads to destruction of most of the mRNA before it can migrate to the cytoplasm. This explains why mRNA precursors that will hybridize with rat albumin cDNA can be found in the hepatocyte nucleus (Esumi *et al.*, 1982), but little or no mRNA for albumin is detectable in the hepatic cytoplasm of Nagase rats.

DNA isolated from fibroblasts of human case 16, an American Indian girl, also showed a mutation at an intron splice location as the cause of her lack of circulating albumin (Table 4-8, residue 214). A single A → G mutation at nucleotide 7706 in the 3' splice site of intron 6 causes failure of exon splicing at this point, and the mRNA again is believed to be destroyed in the nucleus.

A frameshift error has been identified in DNA of human case 10 and in two of her daughters who are heterozygotic for the mutant gene. Insertion of an adenyl base caused a frameshift at amino acid residue 267 in exon 8 (Table 4-8). The frameshift generated a stop codon at residue 273; the mRNA is presumably intact but the albumin produced would be truncated at less than half of normal size. This report was also the first demonstration at the genetic level that analbuminemia is indeed autosomal recessive in nature.

Three other human cases of analbuminemia have now been traced to point mutations that generate stop codons (Watkins *et al.*, 1994b). In cases 3, 15, and 18, C → T mutations yield stop codons at residues 114, 214, and 32, respectively (Table 4-8). Case 3 showed C → T at nucleotide 4446 in exon 4, changing the codon for Arg-114 to the stop codon TGA. The resulting "albumin," if it were produced at all, would be only 113 residues in length.

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TABLE 4-9
Register of Analbuminemia Cases

Case	Initials	Sex	Date of Birth	Age: At time of report	Age: At death	Location	[SA] (mg/L)	Consanguineous	Mutations identified?	Symptoms	Reference
1	F.Br.	F	1922	31	69	Tubingen, Germany	16	+		Edema, fatigue	Bennhold *et al.* (1954)
2	K.Br.	M	1932	23	59	Tübingen, Germany	20	+		None	Bennhold *et al.* (1954)
3	G.M.	F	1923	29	70	Bethesda, Maryland	240	+	+	Edema	Bartter and Waldmann (1959)
4	H.G.	M	1930	29		Lausanne, Switzerland	100	+		Edema	Beck and Dorta (1959)
5	J.L.	M	1894	62	68	Oklahoma City, Oklahoma	30	−		Edema	Shetlar *et al.* (1959)
6	A.M.	F	1908	49	55	Belfast, United Kingdom	1200	−		Edema	Montgomery *et al.* (1966)
7	J.M.	M	1944	21		Ljubljani, Yugoslavia	100	?		Low blood pressure	Mahkota *et al.* (1965)
8	I.Y.	F	1911	55		London, United Kingdom	380	+		Edema	Freeman (1967)
9	A.M	M	1948	25		Okayama, Japan	<10	+		Low blood pressure	Arimori *et al.* (1970)
10	R.R.	F	1942	29		Perugia, Italy	70	+	+	None	Fabiani and Pauluzzi (1971)
11	U.R.	M	1933	38		Perugia, Italy	110	+	+	None	Fabiani and Pauluzzi (1971)
12	H.A.	M	1954	15		Kablia, Algeria	120	+		Edema	Irunberry *et al.* (1971)

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13	Kh.A.	M	1956	13		Kablia, Algeria	680	+		Edema	Irunberry *et al.* (1971)
14	Z.A.	F	1959	10		Kablia, Algeria	50	+		None	Irunberry *et al.* (1971)
15	?	M	1973	<1		Vancouver, Canada	52	+	+	Edema	Cormode *et al.* (1975)
16	J.S.	F	1961	12	32	Seattle, Washington (American Indian)	170	+	+	Seizures	Boman *et al.* (1976)
17	St.Kh.	M	1974	1		Havre, France (Algerian)	64	+		Edema	Goulè *et al.* (1976)
18	M.E.	M	1937	29		Codogna, Italy	50	-	+	Headache	Di Guardo *et al.* (1977)
19	J.J.	F	1960	18		Barcelona, Spain	5500?	?		None	Carretero *et al.* (1978)
20	?	F	1938	6		Ann Arbor, Michigan	8500?	−		Edema	Weinstock *et al.* (1979)
21	S.F.	F	1948	30		Bari, Italy	52			Edema	Dammacco *et al.* (1979)
22	E.Ma.	M	1922	60		Zurich, Switzerland	17	?		Edema	Weigand *et al.* (1983)
23	M.C.	F	1926	54		Nantes, France	265	+		None	Sagniez *et al.* (1982)
24	C.K.	F	1937	43		Basingstoke, United Kingdom	2700?	?		Edema	Platt *et al.* (1985)
25	H.B.	M	1975	10		Port Elizabeth, S. Africa	81	?		Edema	Berger *et al.* (1985)
26	T.H.	M	1966	19		Omaha, Nebraska	<220	?		Seizures	Jolliff and Williams (1986)
27	"3"	F	1968	18		Vilnose, Russia		?		Edema	Belokrinitskaia *et al.* (1985)
28	Y.D.	M	1983	2		Koln, Germany	380	?		Edema	Weigand and Schopf (1981)
			Average:	24	59						

[a]See Table 4-8

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The mutation in case 15 occurred precisely at the start of exon 7, at nucleotide 7708 immediately following the intron 6/exon 7 cleavage site. Splicing was not affected, but the Trp-214 codon, which spans this splice site, changed from TGG to the stop codon TGA. In this case the truncated albumin product would have 213 residues. Watkins *et al.* (1994b) pointed out that the mutation in case 16 occurred just two nucleotides earlier than that for case 15, but within the intronic splice site.

In case 18, a C → T mutation at nucleotide 2368 in exon 3 changed the codon CAG for Gln-32 to the stop codon TAG. The translation product would thus have only 31 residues.

Truncated albumin forms have not been found in the blood, but there is evidence in the rat that some are produced within the hepatic cytoplasm. On close analysis, four species of mRNA were found in the hepatic cytoplasm of Nagase rats: intact mRNA (1/4000 as much as normal) and more abundant species lacking exon G, exons GH, or exons HI (Kaneko *et al.*, 1991).

Immunohistochemical staining of Nagase rat liver showed the presence of albumin in the cytoplasm of a very few cells, $1:10^5$ in neonates but increasing with age or administration of a carcinogen. The albumin in the hepatic cytoplasm was in the secretory channels, and included an abnormal albumin of 60 kDa in addition to a smaller amount of normal 67-kDa albumin. The 60-kDa product was proposed to represent albumin produced without the sequences of exons HI; it could not be found in the circulation and was presumably not secreted from the liver cell.

Mildly truncated albumin forms *are* found in the blood in several types of alloalbuminemia, namely, Bazzano, Rugby Park, Venezia, and Catania (Table 4-8). These are the result of frameshifts near the carboxyl terminus or omission of exon 14, and so are shortened by four to seven residues. This degree of shortening does not create a severely damaged albumin molecule, nor does it affect the disulfide-bonded loop structure. Hence, they may escape detection by the chaperon mechanisms that bind misfolded nascent proteins, whereas more seriously altered albumin forms would be captured and degraded (Chapter 5, Section III.C.2).

What, however, can explain the minute amounts of circulating albumin of normal size and immunologic properties found in both analbuminemic humans and rats? The most probable answer appears to be that the splicing process is very slightly "leaky," as has been demonstrated in the case of a hemoglobin variant (Treisman *et al.*, 1983), and tiny quantities of normal mRNA are produced and reach the cytoplasm.

The widely disperse and very rare occurrence of analbuminemia in humans suggests that it is caused by local independent mutations. The 28 cases given in Table 4-9 arise from 24 families as follows: United States, 6; Italy, 3; Algeria, Germany, Switzerland, and the United Kingdom, 2 each; and Jugoslavia, Japan,

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Canada, France, South Africa, Spain, and Russia, 1 each. The mutant gene would reside unnoticed in a family until intermarriage among its descendants resulted in a homozygous individual. Each is probably unique; the mutations in all five human families so far discovered are distinctive, and Ruffner and Dugaiczyk (1988) showed that the defect in case 16 is *not* present in another case tested, case 22. Physiological implications of analbuminemia are considered in Chapter 5, Section II,C.

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5

Metabolism: Albumin in the Body

Until the 1940s biochemists regarded proteins as stable molecules that endure for the lifetime of their host. Schoenheimer and Rittenberg (1942) at Columbia dispelled this notion in the isotopic tracer experiment by showing that only half of [^{15}N] leucine injected into a rat was catabolized, and the remainder appeared in proteins of active organs and of plasma. Hence synthesis of plasma proteins, including albumin, continues in adulthood as well as during growth.

To maintain a dynamic state, loss must equal gain. Sterling in 1951 established that, even in the steady state, ^{131}I-labeled albumin disappears from the circulation in a first-order manner at about 4% per day. Madden and Whipple (1940) demonstrated that a starving dog could be kept in nitrogen balance by injection of plasma proteins, evidence that the amino acids of these proteins can be used as food.

In the 27 days between its birth and death, a typical albumin molecule makes about 15,000 passes through the circulation, shuttling cargo of various kinds between ports of call in its role as the tramp steamer of the body. And, like any ocean-going vessel, the albumin molecule incurs some damage on its travels, accumulating barnacles in the form of ligands that resist offloading. This chapter will consider the formation of albumin, its distribution, functions, and alterations in the circulation, and, finally, its journey to the scrapyard. A volume edited by Rothschild and Waldmann (1970) is a comprehensive source of further information on albumin metabolism.

I. BIOSYNTHESIS

Although answers have been found to the questions of where, when, how, and how rapidly albumin is formed, albeit with several gaps in the "how," the

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regulation of its synthesis is still far from being understood. Albumin follows the one gene–one protein rule and, being a single-chain protein, requires no assembly of chains. Both alleles of the single gene on chromosome 4 are transcribed to form precursor mRNA. The mature mRNA leaves the nucleus and is translated into a peptide chain by a string of ribosomes in the cytoplasm. As a member of the group of proteins designated for secretion, the nascent albumin quickly passes into the cisternae of the secretory channel where it folds to its native configuration and forms its 17 serial disulfide bonds. There is no storage of newly made albumin awaiting a signal for secretion, only a small amount in transit during its 30-min trip through the parent cell. Mechanisms of this process are discussed below.

A. Systems Used to Study Albumin Biosynthesis

The earliest studies of albumin metabolism were conducted *in vivo*, by measuring changes in circulating albumin concentrations under different conditions; the marked fall in circulating albumin with protein deprivation was an important finding. Much useful information is still obtained through such nutritional studies and through clinical observation.

The availability of isotopic tracers—heavy isotopes in the early 1940s and radioactive nuclides as a benefit of the atomic age in the late 1940s—permitted dissection of biosynthesis from degradation. Albumin and hemoglobin were the first individual proteins to which these tracers were applied for studies of biosynthesis. Although the labeled amino acids initially were of low specific radioactivity, requiring the injection of greater-than-tracer doses, significant information was still accumulated. Friedberg *et al.* at Berkeley (1948) showed the fate of injected DL-[^{35}S]methionine in rats and documented a 30-min delay before its appearance in circulating plasma proteins. In a major study Borsook *et al.* at Pasadena (1950) synthesized six ^{14}C-labeled amino acids and injected them into mice, rabbits, and guinea pigs; they noted the very rapid disappearance of the labeled free amino acids, within 10 min, the lag in appearance in circulating proteins, and, for the first time, the preponderance of activity in the "microsome" fraction of liver cells, vesicles sedimenting at high speed after removal of the mitochondrial fraction. The review by Borsook in 1950 summarized these early studies.

Although the intact animal is the ideal subject for biologists, to determine mechanisms and the effects of regulatory substances such as hormones requires narrowing the field of study. Liver slices, 0.5 mm thick, had been introduced in the study of metabolism by Otto Warburg in 1923. When incubated in 95% oxygen, layers about 12 cells deep (0.18 mm) on both sides remain biologically active; a drawback is the presence of the central zone, about 0.15 mm, which is anoxic. It was in slices that production of albumin was first observed without the

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use of isotopes (Peters and Anfinsen, 1950b). When an isotopic tracer was desired, $^{14}CO_2$ was effective and economical (Peters and Anfinsen, 1950a); low specific radioactivity was not a problem because the pool of bicarbonate in a 5% CO_2–95% O_2 incubation system is large, and the rapid uptake of CO_2 into aspartic and glutamic acids provided intracellular tracer amino acids.

Soon after this L.L. Miller perfected the perfused rat liver system (Miller *et al.*, 1951). Albeit more cumbersome, slower, and less adaptable to the study of regulatory factors than are slices, perfusion avoids damage to the hepatic parenchymal tissue and can be observed for as long as 24 h.

The availability of faster laboratory centrifuges (100,000 *g*) allowed experiments that showed that newly formed albumin could first be detected in the microsome fraction (Peters, 1957); other experiments found that microsomes could actually incorporate radiolabeled amino acids into albumin in a cell-free system (Campbell *et al.*, 1960). The true site of synthesis was then traced back farther to isolated ribosomes, which formed small amounts of labeled albumin without the aid of membranes (Lingrel and Webster, 1961).

Over the next 10 years, *in vivo* pulse-labeling, "grind-and-find" experiments with improved cell fractionation equipment showed that the pathway for new albumin molecules was ribosome → rough endoplasmic reticulum (RER) → smooth ER → Golgi complex → exocytosis (Glaumann, 1970; Peters *et al.*, 1971). The time course is about 25 min for the average albumin molecule in the intact mammalian liver.

Isolated liver cells in culture were employed for the study of albumin synthesis beginning about 1967 (Namba, 1967); adult rat liver cells are difficult to maintain for more than about 2 days, but fetal liver cells (Yeoh *et al.*, 1985) or various hepatoma cell lines (Messina, 1992) are more amenable to culture. They have become popular for measuring effects of hormones and other regulators on transcription as well as on translation and stability of albumin mRNA. With the development of cDNA probes investigators were able in the mid-1980s to locate and quantify albumin mRNA by *in situ* hybridization (Bernuau *et al.*, 1985). The techniques of cell fusion, gene insertion, and creation of transgenic mice have now all been applied to the study of albumin production with fruitful results.

Table 5-1 compares the features of various systems in which albumin biosynthesis can be studied. The farther the system from the intact animal, the more disruption of normalcy and the lower the rate of albumin production. Even *in vivo* it is impossible not to disturb the animal by the effects of anesthesia (barbiturates quickly bring changes in microsomal enzymes) or stress.

Both slices and cell cultures permit the study of many conditions and regulators on tissue samples from a single animal. Cultured cells are "cleaner" than liver slices, but require tedious effort to produce gram quantities. Neither slices nor isolated cells are ideal for studying the progression of newly formed albumin through cytoplasmic organelles. Their cell walls appear to have toughened and

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TABLE 5-1
Features of Systems to Measure Albumin Biosynthesis[a]

System	Albumin production [mg/g ww/h estimated (rat)]	Departure from ideal[b]	Observation time (h)		Flexibility for controls and variations	Ability to make subcellular organelles	Reference
			Maximum	Minimum			
Intact animal	0.8	—	—	—	Low	Best	Peters and Peters (1972)
Pulse label		±	1	0.2	—	—	Olufemi *et al.* (1990)
Constant infusion or flooding	—	+	72	6	—	—	Ballmer *et al.* (1990)
Perfused liver	0.7	++	24	0.5	Low	Fair	Miller *et al.* (1990)
Liver slices	0.3	+++	6	0.01	High	Poor	Peters (1973)
Cultured cells	0.3	++++	96+	0.01	High	Poor	Dahn *et al.* (1993)
Subcellular fractions	0.1	+++++	1	0.01	High	—	Campbell and Kernot (1962)

[a] Adapted from Peters (1983).
[b] ±, minimal, to +++++, maximal.

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require more strenuous homogenization to break open compared to cells of intact liver (Krack *et al.*, 1980) (and the author's experience), and the time of passage of albumin through the organelles is significantly slower in slices and isolated cells (Edwards *et al.*, 1976) than the ≈ 25-min period observed in the intact liver (Peters and Peters, 1972).

How to isolate albumin from a test system? In my first labeling studies, I used a variation of the classic Cohn cold alcohol techniques (Peters and Anfinsen, 1950a); the product was pure but the method tedious and the yield imperfect. The shortcut isolation with TCA–alcohol (Debro *et al.*, 1957) is useful when dealing with a relatively pure source such as an incubation medium, and preparative electrophoresis and ammonium sulfate fractionation procedures have also been applied.

Immunochemical techniques are the most useful and the most specific. The albumin can be precipitated directly by antialbumin or the precipitation can be aided by staphylococcal protein A, which binds to immunoglobulin G. A precaution, often overlooked, is that radioactive complement is synthesized simultaneously by the liver; it may couple to these immune precipitates and falsely elevate the counts obtained. To remove this interferent a "clearing" step, pretreatment with an unrelated antigen–antibody system such as chicken serum albumin or ovalbumin, is generally recommended (Lingrel and Webster, 1961; Peters, 1962b).

A recent shortcut has been to perform SDS-gel electrophoresis directly on cell extracts, identifying the albumin by immunostaining of the resultant patterns. It has the advantage of confirming the relative molecule mass of the albumin molecule, but suffers in terms of quantitation, particularly when coupled with autoradiography to determine incorporation of radioactivity.

B. Expression (Transcription)

1. Sites of Albumin Expression

That the liver is the chief if not the sole source of albumin had been presumed from the common finding of hypoalbuminemia with hepatic cirrhosis. Experimenters in the preisotopic era noted that the circulating albumin levels fell sharply in the totally hepatectomized dog or rat; this could hardly be accepted as proof considering the accompanying trauma, but demonstration that whole-body incorporation of [^{35}S]methionine into albumin fell essentially to zero on removal of the liver was more convincing (Tarver and Reinhardt, 1947). Direct evidence of the ability of the liver to produce albumin had already appeared—production of albumin by surviving slices of chick liver and uptake of [^{14}C]lysine into albumin in the perfused rat liver (see Table 1-1).

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Albumin can be visualized within the cytoplasm of liver cells by antialbumin antibodies carrying a fluorescent or peroxidase label. When observed at the electron microscopic level, using electron-dense markers, the albumin lies within the cytoplasmic secretory channels so it is presumed to be newly manufactured (Guillouzo *et al.*, 1982). Under normal conditions nearly all liver cells contain this albumin; earlier reports of a spotty distribution of albumin-containing cells were apparently the result of inadequate removal of blood from the liver before fixation or lack of stirring on fixation of small pieces of tissue (Brozman, 1971; Horikawa *et al.*, 1976; LeBouton and Masse, 1980). Uptake of radiolabeled amino acids is more active in periportal cells when the tracer is injected in the portal vein (Schreiber *et al.*, 1970). Conversely, after an overnight fast there is more albumin in perivenous than in periportal cells (LeBouton, 1982), probably the result of breakdown of upstream liver tissue to form free amino acids. All of the lobes of the liver appear to participate evenly, with the exception that amino acids arriving from the small intestine are more actively incorporated into proteins by the left lobe of rat liver (Muglia and Locker, 1984).

When evidence of transcription of the albumin gene was sought in the adult mammal, by hybridization of DNA probes with total RNA extracted from adult rat tissues, mRNA for albumin was readily observed in liver; Sell *et al.* (1985) reported finding no transcripts in other organs (kidney, brain, heart, lung, or intestine), but Nahon *et al.* (1988), by a similar procedure, found minute quantities of mRNA in kidney and pancreas. The small amount in pancreas appeared to have a polyadenylate tail of normal length, whereas the mRNA from kidney had a short tail or none at all. There has been no evidence that the mRNA in these nonhepatic tissues is translated, in agreement with studies cited above on the lack of circulating albumin synthesis in the liverless dog or rat.

Whether serum albumin found in milk is produced by the mammary gland or derived from the circulation has not been resolved. Phillippy and McCarthy (1979) infused [^{14}C]leucine into either the teat or a peripheral vein in goats and concluded that at least 10–20% of milk serum albumin is made in the mammary gland. Jordan and Morgan (1967), on the other hand, felt that serum albumin in milk of the rat arose only from the plasma.

Skeletal muscle of the chick, however, does appear to synthesize a minute amount of protein identical to serum albumin, according to two persuasive studies on incorporation of labeled amino acids (Muller and Heizmann, 1982; Kobayashi and Tashima, 1990). Its synthesis accelerates in muscle hypertrophy (Yamada *et al.*, 1984), but it is never secreted from the muscle cell. Intracellular albumin has also been identified in the thyroid gland; no albumin mRNA is detectable in gland tissue (de Vijlder *et al.*, 1992), and the intracellular albumin does not incorporate labeled amino acids *in vitro*, so it presumably enters from the plasma (Thomas-Morvan and Ceriani, 1977).

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2. Ontogeny of Albumin Superfamily

Expression of albumin during fetal development is intertwined with that of the other members of its superfamily, AFP, ALF, and DBP. True to its name, α-fetoprotein is the first of the family to appear. mRNA for AFP is detectable in the yolk sac of the fetal rat between 10 and 12 days of development, peaking in concentration about day 17 (Tamaoki *et al.*, 1974; Cooke *et al.*, 1991). Translation of AFP from this mRNA occurs at this time, as judged by incorporation of radiolabeled amino acids (Gitlin and Kitzes, 1967). mRNAs for albumin and DBP are in the yolk sac at much lower levels, detectable only following amplification by polymerase chain reaction (PCR) (Cooke *et al.*, 1991). However, albumin was found by immunohistochemistry in the cells of the yolk sac of a 20-day human conceptus (Jacobsen *et al.*, 1981), and its synthesis by the 17-day chick yolk sac and the 5.5-week human fetus has been reported (Gitlin and Kitzes, 1967; Gitlin and Gitlin, 1975).

In the liver AFP is likewise the family forerunner; its mRNA appears between 12 and 14 days in rat and mouse (Tamaoki *et al.*, 1974; Muglia and Locker, 1984). The mRNA level peaks about day 16 and declines sharply by birth. mRNAs for albumin and DBP (Cooke *et al.*, 1991), conversely, rise in concentration only after day 12 and maintain a plateau after about 18 days. ALF message trails those of albumin and DBP, being barely detectable in fetal liver and strongly induced postnatally (Bélanger *et al.*, 1994).

Translation of AFP in rat liver subsides after day 13 whereas that of albumin rises (Tamaoki *et al.*, 1974). Human liver can synthesize albumin and AFP at 4 weeks of gestation (Gitlin and Gitlin, 1975).

mRNAs for AFP and albumin are seen in the intestine in early fetal life (Sell *et al.*, 1985; Naval *et al.*, 1992); translation only of AFP at low levels has been noted (Gitlin and Gitlin, 1975; Shi *et al.*, 1985). The Gitlins (father and son) further observed that AFP in the fetal shark is first made by the stomach, then the yolk sac, and finally by the liver, the site of synthesis moving caudally during embryogenesis. mRNAs of both AFP and albumin, but not their translation, have been detected in other fetal organs: kidney, pancreas, heart, and lung.

Appearance of *circulating* albumin in the fetus parallels that of DBP but is reciprocal to that of AFP. In man, circulating AFP is detectable at 67 mg/L at 6.5 weeks of gestation, rising rapidly to 3 g/L by 10–13 weeks and falling to 10–100 mg/L at term. Circulating albumin is about 1.5 g/L at 6 weeks and reaches adult levels (≈35 g/L) by 40 weeks (see Table 6-1, Chapter 6, for further details); albumin does not cross the placenta from the mother in significant amounts, so this albumin represents synthesis by the fetus. DBP is detectable at 70 mg/L at 12 weeks and rises to 230 mg/L, about half the adult level, at term; its production by the fetus is evident from frequent differences between fetal and maternal DBP phenotypes (Gitlin and Gitlin, 1975).

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The marked change in albumin expression during metamorphosis of amphibians (Chapter 4, Section III,A,1) is traceable to a marked acceleration of transcription, evoked by an abrupt rise in thyroid hormone levels (Schultz *et al.*, 1988; Averyhart-Fullard and Jaffe, 1990). The albumin is the same protein as judged by chemical properties (Feldhoff, 1971).

3. Process of Transcription

The two strands of the inactive DNA double helix are coiled around histone cores, forming a string of nucleosomes. A binding site for histone H1 in particular has been identified at 300–400 bp into the 5' end of the albumin gene (Sevall, 1988). In the tight configuration the DNA is protected from attack by nucleases. The helix must uncoil at least locally and the two strands must separate in order for the transcribing enzyme, RNA polymerase II, to gain access. This conformational change of the DNA helix is controlled by a series of soluble, *trans*-acting regulatory proteins that act by binding at built-in *cis*-acting sequences termed *promoters* or *enhancers* at the 5' end of the gene.

These elements are classified as proximal or distal elements, depending on their distance in the 5' direction from the Cap point of the gene. The most important of these enhancer regions for the albumin superfamily occur in the 250 residues adjacent to the Cap point, and are diagrammed in Fig. 5-1. Some of the more distant ones can be seen in the gene sequence of Fig. 4-2.

Close to the Cap site is the "TATA box," a recognition site for the transcribing enzyme, RNA polymerase II. It is seen as TATATTA ending at bp –26 (Fig. 5-1). The binding site of the enzyme actually extends into exon 1 and overlaps the first codon.

The first of the promoters is the sequence ending at bp –52, AGTTAATAATCTA, termed the proximal element (PE); it is the purported recognition site for the important liver regulatory protein, HNF1 (Tronche and Yaniv, 1992) (earlier termed LFB1, HNF1α, HP1, or APF). HNF1 is described as a variant homeodomain, with an amino acid sequence that is highly conserved among mammals (94% between HSA and RSA). Among the plasma proteins it regulates are transthyretin, α_1-antitrypsin, and fibrinogen (Costa *et al.*, 1988). Like some other factors, it attaches to the DNA as a dimer; this is in keeping with the semipalindromic nature of the PE sequence to which it binds (Fig. 5-1).

The configurational change brought about by the attachment of HNF1 is apparently sufficient to promote a minimal level of transcription, such as occurs for AFP and albumin in the early fetus. *Trans*-factors acting on the more distal elements increase ("enhance") this production markedly.

Next in line (Fig. 5-1) is the CAT box, named for its usual sequence, and found in some form in all members of the superfamily. It binds the ubiquitous regulatory factor, NFY (Mantovani *et al.*, 1992); it also attaches to the gene for

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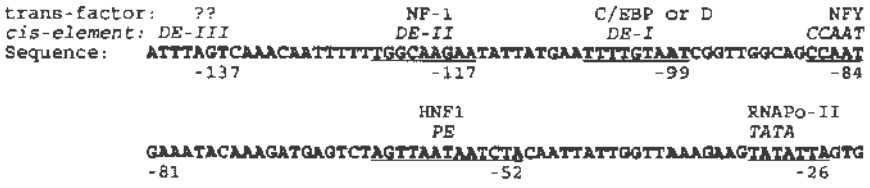


Fig. 5-1. Location of TATA box and some promoter *cis*-acting sequences regions in the near-5' end of genes for the albumin superfamily. DE, Distal element; PE, proximal element. Other labels are described in the text. Information from Sakai *et al* (1985), Minghetti *et al* (1986), Herbomel *et al*. (1989), and Witke *et al*. (1993).

collagen. Just 5' to the CAT box is the sequence, TTGGC, which acts as a promoter in eukaryotic genes.

Acting at distal element (DE) I, ending at –99, are two *trans*-factors, C/EBP (enhancer binding protein) and D. C/EBP is reported to be important early in development, whereas D expression is maximal after cell division has ceased (Mueller *et al.*, 1990). The *trans*-factor, NF1, binding at distal element II, has a minor role in enhancing transcription. A "silencer" has been identified at –10.5 to –8.5 kb; the C/EBP region can override it (Herbst *et al.*, 1989). C/EBP is now believed to act synergistically by interacting with a Pro/Gln-rich domain in HNF1 (Wu *et al.*, 1994). Its leucine zipper appears to have a role in determining which cell types will express albumin (Nerlov and Ziff, 1994).

HNF1 is reported to bind also at several sites upstream, at bp –486 (Tronche *et al.*, 1989), and more remotely, at –1.7 kb (Hayashi *et al.*, 1992). Another unidentified regulator protein binds at –6 kb. Putative progesterone receptor sites occur at +1 to +19 within exon 1, and at –38 to –56, –610 to –628, and –784 to –802 bp in the nontranscribed 5' region (Urano *et al.*, 1986). A negative regulatory region is proposed to lie between by –486 and –673 (Frain *et al.*, 1990). Another TATATTA sequence, unaccompanied by a CAT box, lies upstream at –793 in the human gene; it may represent a relic promoter. The enhancer sequence region is highly conserved among human, rat (Sargent *et al.*, 1981a), and chick (Hachè *et al.*, 1983) albumin genes, to about bp –250.

The activation of these enhancers, plus probably several at more distal sites, is believed to be sufficient to permit transcription from the albumin family genes at high rates. The extracellular matrix is also significant. Production of the 42-kDa C/EBP protein factor in isolated rat hepatocytes is accelerated by growth on a matrix of basement membrane, causing differentiation of the cells into a rounded shape and increased transcription of the genes for albumin, transthyretin, and other characteristic liver products. A matrix of collagen, on the other hand, activates the widely spread mammalian transcription factor, AP-1, which binds to

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sites in the remote albumin enhancer region and promotes DNA replication and growth rather than differentiation (Rana *et al.*, 1994). The *ras* gene product also promotes AP-1 activity, transfection with normal c-Ha-*ras* inhibiting albumin transcription (Hu and Isom, 1994).

The fall in AFP transcription that begins *in utero* and reaches near zero at birth is partly brought about by a silencer at −1.8 kb in the 5' region of the AFP gene (Nakabayashi *et al.*, 1991). Involvement of the G protein signal transduction pathway is intimated by the down-regulation of the AFP gene by oncogenic *ras* (Nakata *et al.*, 1992). A major negative factor in AFP regulation is hormonal; glucocorticoids binding at the glucocorticoid receptor element (GRE), TGTCTA, interfere with the activity of other enhancers and turn off AFP transcription almost completely (Zhang *et al.*, 1991; Bernier *et al.*, 1993). Corticosterone, for instance, begins to rise at day 14 in the rodent embryo and peaks shortly after birth.

With the release of enhancer proteins from the AFP 5' region, which we recall is the intergenic stretch between albumin and AFP, these enhancers are proposed to shift to the albumin 5' region and stimulate albumin transcription in the adult (Nakata *et al.*, 1992). This reciprocal interplay has raised the conjecture that the sequential clustering of the albumin and AFP genes may have a function rather than being a mere consequence of evolution (Camper *et al.*, 1989). AFP transcription can proceed independently of the presence of the albumin gene (Bernier *et al.*, 1993), but expression of *albumin* (in transgenic mice) requires several *AFP* enhancers.

Less is known about the regulation of DBP and ALF, the other members of the superfamily. The elements thus far identified in the DBP 5' region are only weakly homologous to those of albumin, and the relative location of its gene remains unidentified. The order of activation of the superfamily appears to be AFP–albumin–DBP–ALF, whereas their order in the multigene locus is albumin–AFP–ALF–(DBP?). Bélanger *et al.* (1994) suggest that the albumin superfamily multigene locus escapes the polarity rule followed by other developmentally regulated multigene families, by which gene activation occurs sequentially according to their linear position.

The unfolding of the DNA double helix brought about by the above regulatory factors causes increased susceptibility of specific sites in the 5' region to cleavage by DNase I (Kunnath and Locker, 1985). Three sites have been identified in the rat for albumin and AFP (Nahon *et al.*, 1988), and two for ALF (Bélanger *et al.*, 1994), usually close to a promoter sequence. Only one of the AFP sites is detectable in adult liver or in kidney.

The more open structure also correlates with loss of methyl groups from 5-methylcytosine in certain specific 5'-CG pairs. Thus there is an inverse relationship between level of expression and degree of methylation (Nahon, 1987). Whether the loss of methyl groups is a cause or effect of the conformational change during transcription is uncertain.

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Transcription begins when the RNA polymerase II binds the plus strand of the opened DNA helix at the TATA box, extending into exon 1. It copies the strand, including introns and exons and an extensive portion of the 3' region, into a complementary messenger RNA. In the mRNA, uridine (U) replaces thymidine (T) in pairing with cytosine (C). The 5'-terminal AGC sequence of the growing pre-mRNA transcript is "capped" by addition, 5' to 5', of guanosine triphosphate, which becomes methylated at its N-7 position; this group protects against RNase activity and later acts as a signal for the ribosomal translation mechanism.

Polyadenylation of the 3' end rapidly follows. This process begins with cleavage of the RNA chain at the UCU/AA sequence shortly after the AAUAAA signal at bp 16,940 in the gene sequence, 165 bp beyond the carboxyl-terminal codon, followed by action of a poly (A) polymerase to attach 50–250 adenyl nucleotides (Sachs and Wahle, 1993). Another functional cleavage site occurs about 150 bp farther downstream on the pre-mRNA at bp 17,095. The large (26S) pre-mRNA for albumin has been detected at this stage in rat liver nuclei (Strair *et al.*, 1978).

The introns (Fig. 4-1) are then removed, by cleavage at their 5'-(GUA/G) and 3'-(C/UAG) sites, and the exons spliced together to form mature mRNA. The mature mRNA enters the cytoplasm through a nuclear pore, where it is available for translation into protein by ribosomes (Fig. 5-2). Figure 5-3 shows graphically the pairing of mature albumin mRNA with the albumin gene; the exons bind, and the intronic regions appear as excluded loops.

Several reviews of the process of transcription of the albumin and AFP genes and its regulation are recommended (Nahon, 1987; Camper *et al.*, 1989; Papaconstantinou *et al.*, 1990).

4. Control at Transcriptional Level

The rate of albumin production can be regulated in several ways. Synthesis (translation) requires mRNA, a supply of amino acids activated by binding to transfer RNA (tRNA), the ribosomal machinery for assembly, and energy in the form of GTP or ATP. The level of mRNA in the cytoplasm is the result of its transcription and its degradation, and factors have been found that affect both of these processes. This section is concerned only with factors that appear to regulate *synthesis* of albumin by affecting the rate of mRNA transcription from the albumin gene.

a. Trauma and Disease. Clinicians and medical laboratorians are aware that a decline in the circulating albumin level is a common feature in disease, and that a normal albumin concentration in blood is one of the most frequently tested indicators of health. The albumin/globulin (A/G) ratio, although a crude measurement, still has widespread application.

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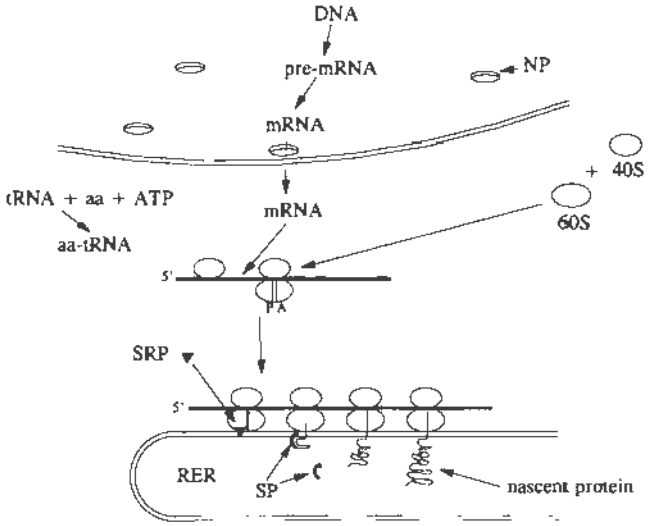


Fig. 5-2. Schematic pathway of translation of a protein destined for secretion. NP, Nuclear pore; aa, amino acid; SRP, signal recognition particle; RER, rough endoplasmic reticulum; SP, signal peptide.

This close linkage with a healthy condition suggests that factor(s) present in disease directly affect the formation of albumin. In the **acute-phase reaction,** i.e., the generalized response of the body to a stressful insult—trauma, a burn, or an acute infection, the concentration of the albumin mRNA is depressed (Cairo *et al.*, 1982; Liao *et al.*, 1986), as is that of several other "negative acute-phase proteins" such as transferrin and transthyretin. A turpentine-induced abscess, for example, causes an 80% fall in albumin mRNA in rat liver within 20 h (Milland *et al.*, 1990). Acute liver disease is associated with decreased albumin mRNA in human liver biopsy samples (Ozaki *et al.*, 1991). Conversely, plasma proteins important for defense, such as fibrinogen and haptoglobin, show an increase in mRNA concentration in the acute-phase reaction.

The falling albumin mRNA level has been linked to a deceleration of transcription (Milland *et al.*, 1990; Ozaki *et al.*, 1991); whether a concomitant acceleration of mRNA degradation is a contributing factor is discussed later (Section I.E). The actual regulatory agent appears to be one of the **cytokines.**

Acute-phase cytokines are messenger molecules released by monocytes that travel through the bloodstream and alert the body's organs of defense on injury or acute disease. If administered *in vivo* they cause a fall in albumin level and in

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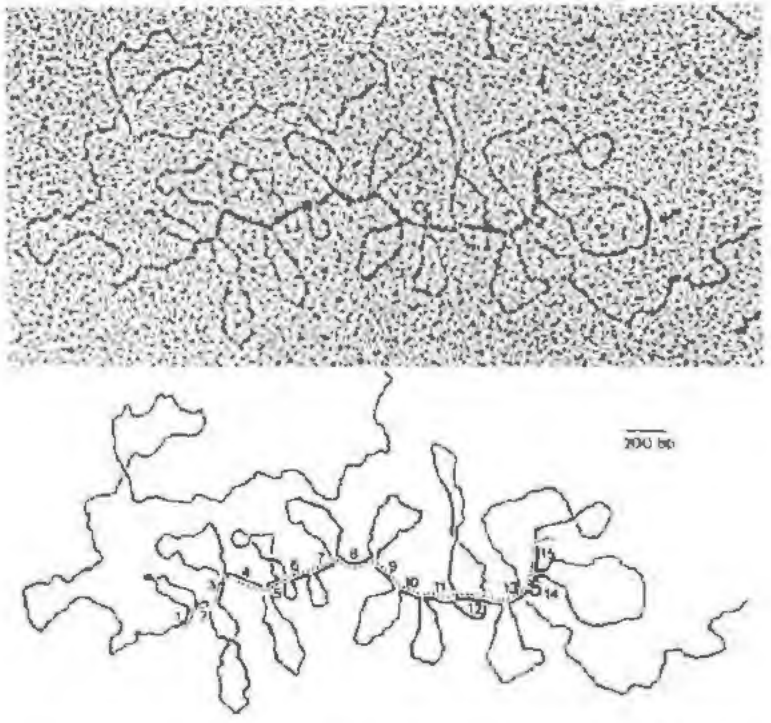


Fig. 5-3. Electron micrograph and interpretive diagram of an artificial hybrid of mature mRNA of albumin and the DNA strand of its gene. This preparation is of the 68-kDa albumin of *Xenopus laevis*. mRNA is indicated as dots (•••••) and cloned DNA as a solid line (—). The 15 exons are numbered; DNA regions coding for introns can be seen ballooning out from the mRNA. The 5' end of the DNA is to the left and the 3' end is to the right. Reproduced from May *et al.* (1983) by permission of the authors and Academic Press Ltd. (London).

albumin synthesis (Moshage *et al.*, 1987), the latter effect being seen in cultured hepatocytes as well (Koj *et al.*, 1984) and traced to an inhibition of transcription (Moshage *et al.*, 1988). The major cytokine that affects hepatic protein synthesis is **interleukin-6 (IL-6)**, a third-level mediator of the acute-phase reaction (Ramadori *et al.*, 1988). After 20 h IL-6 caused a 50% depression of albumin transcription in human hepatocytes in culture (Castell *et al.*, 1990). A *cis*-acting site has not been recognized for IL-6, and there is evidence that its down-regulatory action occurs through loss of the liver-specific *trans*-acting factors HNF1 and D-site protein (see Section I,B,3).

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Mice transfected with cells producing **tumor necrosis factor** (TNFα) became cachectic, and showed a marked (90%) drop in albumin synthesis and mRNA level traceable to down-regulation of albumin gene transcription (Brenner *et al.*, 1990). The effect occurred before weight loss set in and seemed specific for albumin expression. Human subjects receiving a 28-day infusion of TNFα at doses up to 25 μg/m²/day, however, showed no change in levels of circulating albumin or other nutritional marker proteins (Hardin *et al.*, 1993).

Down-regulation of albumin transcription is also seen in hepatoma cells. In these tumors (Andrews *et al.*, 1982; Selten *et al.*, 1982), as well as in hepatocytes following carbon tetrachloride injury (Panduro *et al.*, 1986; Lemire and Fausto, 1991), transcription of AFP is stimulated reciprocally to that of albumin in a manner reminiscent of the fetal state.

b. Hormones. Removal of the pituitary gland, the adrenals, or the thyroid glands from a rat decreases its circulating albumin level in decreasing degree (Levin and Leathem, 1942). Because of the myriad interactions of hormones within the body, however, more direct evidence must be sought in order to decide whether a hormone affects albumin synthesis specifically and whether it acts by control of transcription or at some other point.

Deficiency of **insulin,** or diabetes mellitus, is accompanied by a decrease in albumin synthesis in rats and humans; in diabetic subjects albumin synthesis was depressed to a daily replacement rate of 7.0%, which rose to 9.9% after 6 h of an insulin infusion (De Feo *et al.*, 1991). Perfused livers of diabetic rats show about a 75% reduction in albumin release (Peavy *et al.*, 1978), and the amount of albumin mRNA in the liver is reduced about 75%. Mere addition of insulin to the perfusate does not restore activity in these short-term experiments, rather the insulin must have been given *in vivo*.

A stimulatory effect of added insulin *in vitro* in longer experiments was recognized in rat liver cell cultures as early as 1978 (Dich and Gluud, 1975), and shortly thereafter in chick liver cell cultures (Plant *et al.*, 1983). The deficiency in the diabetic rat was subsequently traced to a 50% drop in the rate of albumin gene transcription (Lloyd *et al.*, 1987), and evidence was obtained for the presence of an inhibitor of albumin promoter activity in diabetic mouse hepatonuclear preparations (Wanke and Wong, 1991). Insulin is now routinely added to hepatocyte cultures as a growth factor, usually accompanied by growth hormone and sometimes steroids.

Not all studies of effects of insulin have yielded the same result. Davis *et al.* (1988) reported that albumin mRNA levels are unaffected when insulin is given to diabetic rats, and Nawa *et al.* (1986) found no effect of added insulin on transcription of the albumin gene in rat hepatocytes. Insulin actually showed a negative effect on albumin transcription in cultured cells of the rat hepatoma H4-IIE (Straus and Takemoto, 1987; Messina, 1992).

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The effect of hypophysectomy can be seen in perfused liver and cultured rat hepatocytes (Ulrich *et al.*, 1954; Griffin and Miller, 1974; Feldhoff *et al.*, 1977). Added **growth hormone** partially restores the rate of albumin synthesis in these *in vitro* systems. It acted to stimulate the rate of transcription in normal rat hepatocyte cultures (Johnson *et al.*, 1991), although degradation of albumin mRNA was also increased.

Adrenocortical hormones have diverse effects on albumin synthesis, and whether they actually control albumin transcription is controversial. Unlike AFP, for which glucocorticoids have a negative regulatory effect on transcription, actions of these hormones on albumin synthesis are neither simple nor direct.

Adrenalectomy is accompanied by a fall in albumin synthesis *in vivo;* in Cushing's disease albumin degradation is found to be increased and synthesis increased in a compensatory manner (Sterling, 1960). Likewise, administration of corticoids to dogs stimulates albumin degradation after a day or more (Takeda, 1964). Jeejeebhoy *et al.* (1972a) helped to resolve the situation by showing that injected cortisol depressed albumin synthesis in rats for the first 3 h but stimulated it after 24 h. The effect was related to the availability of free amino acids to the liver; if extra amino acids were provided with the cortisol the depression of albumin synthesis was not seen. A transient stimulation of albumin synthesis between 3 and 12 h after initiation of the acute-phase reaction may be the result of endogenous steroids, because it is not seen in the absence of the adrenal gland (Majumdar *et al.*, 1967; Koj and McFarlane, 1968).

In the perfused liver system addition of cortisol in the presence of an adequate amino acid supply elevates albumin production by 10% (John and Miller, 1969), about the same increase as that caused by addition of insulin plus growth hormone. Maximal synthesis was obtained when both insulin and cortisol were present.

In cultured liver cells added corticosteroids cause some increased albumin synthesis, which with added insulin rises maximally and is sustained for some days (Hutson *et al.*, 1987; Grunnet *et al.*, 1988). Injection of dexamethasone enhances neither cell-free synthesis nor albumin mRNA concentration in normal rats, but when added to adult (Moshage *et al.*, 1985) or fetal (Yeoh *et al.*, 1985) hepatocyte cultures prevents the normal loss of albumin mRNA over 1 to 4 days.

Nawa *et al.* (1986) have provided the most direct evidence that corticoids affect albumin expression. Their data show that injected hydrocortisone increases transcription of the albumin gene in rats, and dexamethasone at 0.1 μM added to rat liver cell cultures induces transcription, as measured directly in isolated nuclei, the effect peaking at 2 h. By study of the rat gene 5' region they observed that the human sequence –65 to –53, TAGTTAATAATCT (Fig. 5-1), and the homologous rat sequence –63 to –50, TGGTTAATGCCT, resemble GRE- binding regions found in some other proteins and may represent the site for corticoid action. The sequence does not closely resemble the GRE for AFP, TGTCTA, however.

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Corticoids thus are seen to modify albumin synthesis in numerous experimental systems. Their action is complex, being both time and amino acid dependent, and they affect albumin degradation as well as production *in vivo*. They seem to be most effective when given together with insulin, which may reflect the anabolic activity of insulin in promoting both amino acid uptake by hepatocytes and phosphorylation of a critical component of the translation mechanism. Further study of their effect on transcription will be of interest.

Removal of the **thyroid** gland causes a 20% (Griffin and Miller, 1974) to 50% (Peavy *et al.*, 1981) slowing of albumin synthesis by the perfused rat liver or by cultured liver cells. The inhibition affects other secretory proteins (plasma proteins) as well, and is not accompanied by a change in the amount or activity of albumin mRNA. Perfused livers from hyperthyroid rats showed a small stimulation, about 10%. Nawa *et al.* (1986) found no effect of added T3 on transcription of the albumin gene in adult rat liver cells.

In fetal mouse liver cells, T3 stimulated mRNA and protein production for albumin but decreased it for AFP (Anteby *et al.*, 1993); this effect was not confirmed in fetal rat cells, where T3 lowered albumin production (Baranova *et al.*, 1990). A very slow response was seen in cells from a rat hepatoma: a 4-h exposure to 10 n*M* T3 resulted in a fourfold increase in albumin synthesis 15 days later, accompanied by a threefold increase in albumin mRNA level (Conti *et al.*, 1989). AFP responded oppositely, with an eightfold decrease in synthesis and a threefold decrease in its mRNA.

Much greater effects are seen in other experimental models. Tadpoles of *Rana catesbiana* (Schultz *et al.*, 1988) or *Xenopus* (Moskaitis *et al.*, 1989) can be stimulated to premature transcription of the albumin gene by as little as 0.01 n*M* T3, resulting in a sevenfold increase in albumin mRNA level. Hence it appears that the thyroid is an important regulator of albumin production only in larval metamorphosis and perhaps in malignant states.

Retinoic acid had no effect by itself on albumin synthesis by adult rat hepatocytes, but attenuated a stimulatory effect of another agent, epidermal growth factor (EGF) (see below). In two hepatomas it acted oppositely; it enhanced transcription of the albumin gene in rat tumor McA-RH8994 (Wan and Wu, 1992), but depressed it severely in human tumor Hep-3B after 8 h of exposure (Hsu *et al.*, 1992). In both cases the apparent action of retinoic acid was blocked by the transcription inhibitor, actinomycin D. Oddly, there was a marked stimulatory rather than depressant effect on synthesis of transferrin, a companion negative acute-phase protein.

Butyrate, a colonic bacteria fermentation product that can act as an antiproliferative agent in a variety of cancer cells, at <1 m*M* in cell culture elevates the concentration of albumin mRNA. At the same time it reduces the expression of AFP in a reciprocal fashion by repressing the AFP promoter (Tsutsumi *et al.*, 1994).

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Estrogen does not affect albumin transcription but acts by modifying the stability of its mRNA (see Section I,E).

Growth factors, hormonelike cytokines that are only beginning to be explored, have varying effects on albumin synthesis via transcriptional control. **Hepatic growth factor (HGF)** seems to be the most effective, increasing albumin synthesis 40–60% in rat hepatocyte cultures in which the cell growth is dense enough so that cell-to-cell contact is maintained (Takehara *et al.*, 1992). **Epidermal growth factor (EGF)** yielded a 20–30% increase, which was opposed by retinoic acid (Ikeda and Fujiwara, 1993). In fetal rat hepatocytes EGF was stimulatory if the cells were proliferating; maximal albumin synthesis, accompanied by elevation of its mRNA, was obtained if noradrenalin or other agents to increase cyclic AMP levels were also present (de Juan *et al.*, 1992).

Other growth factors observed to stimulate albumin or plasma protein synthesis in normal liver cell cultures are **insulin-like growth factor (IGF), transforming growth factor (TGF-α),** and **acidic fibroblast growth factor** (Takehara *et al.*, 1992). **TGF-β , basic fibroblast growth factor,** and **interleukin-1β** were without effect.

Administration of **granulocyte–macrophage colony-stimulating factor (GM-CSF)** to 14 humans with aplastic anemia significantly lowered circulating albumin levels (Kaczmarski and Mufti, 1990; Takahashi *et al.*, 1991). In this *in vivo* setting, however, GM-CSF enhances production of IL-1 and TNFα, which are known to result in depression of albumin synthesis.

c. Physiological Factors. i. AMINO ACIDS. Albumin synthesis is exquisitely sensitive to an adequate supply of amino acids, as seen *in vivo* by the hypoalbuminemia and edema of kwashiorkor, and *in vitro* by the marked depression of albumin biosynthesis in liver slices and isolated liver cells. The reduction is traceable to a parallel 50–60% decline in albumin mRNA activity (Pain *et al.*, 1978) or mRNA concentration (Sakuma *et al.*, 1987) in livers of rats on 0–4% protein diets. The decline in mRNA is part of a coordinate response to protein malnutrition affecting also transthyretin and other plasma proteins used as nutritional markers (Straus *et al.*, 1994). No change was found in the rate of albumin gene transcription, however (Straus and Takemoto, 1990), so the loss of mRNA apparently occurs through more rapid degradation rather than increased synthesis.

ii. COLLOID OSMOTIC PRESSURE (COP). The logical regulator for albumin synthesis is the circulating albumin concentration itself, providing down-regulation by negative feedback as the desired level is attained. Evidence for such a simple mechanism has been elusive. Albumin synthesis is indeed stimulated by hypoalbuminemia in the case of major losses of albumin from the body—protein-losing enteropathy, for example. Conversely, albumin synthesis is depressed when albumin is given intravenously (Oratz *et al.*, 1970), but almost any mildly traumatic experimentation can call to arms the acute-phase reaction and depress albumin

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production. That administration of other macromolecules, dextran and γ-globulin, has effects similar to the administration of albumin pointed to the colloid osmotic pressure, or the oncotic effect of albumin, as the function on which its regulation is based.

Neither incubated liver slices (Peters, 1973) nor isolated cells (Schmid *et al.*, 1986) show a response of albumin synthesis rate to the COP in a 4-h period. The perfused liver does appear sensitive to dextran or albumin levels in the perfusate (Tracht *et al.*, 1967; Rothschild *et al.*, 1969; Dich *et al.*, 1973), but more specific evidence now is appearing from other systems.

The increase in albumin mRNA concentration seen in the hypoalbuminemia of nephrosis can be blocked by administration of actinomycin D to prevent transcription (Pedraza-Chaverri and Huberman, 1991). A 50–60% decrease in rate of gene transcription of the albumin gene, but not of many other proteins tested, is known to accompany intravenous albumin administration (Pietrangelo *et al.*, 1992).

These authors also used an experiment provided by nature and showed that the rate of transcription of the faulty albumin gene in analbuminemic rats is double that in normal rats. The lack of albumin in these animals is partly compensated by increases in other plasma proteins, but their COPs are still only about one-half of normal, about 12 mm Hg, which apparently provides the stimulus to transcription. When sufficient albumin was administered to correct the pressure deficit, about 16 g/L, the transcription rate of the albumin gene fell to normal by 24 h.

Other evidence comes from hepatocyte cultures. Dextran supplementation of the culture medium of human hepatoma HuH-7 cells down-regulates albumin transcription; the degree of repression is related to the osmotic effect rather than the dextran concentration, smaller dextran particles at the same concentration giving a greater effect owing to their greater number (Yamauchi *et al.*, 1992). When transcription was assessed by linkage to the chloramphenicol acetyltransferase (CAT) expression marker, either albumin or dextran repressed promoter activity of both albumin and AFP; transcription returned to normal 48 h after removal of the albumin or dextran (Tsutsumi *et al.*, 1993).

Pietrangelo and Shafritz (1994) have recently shown that the *trans*-acting liver factor HNF1 is decreased by 50% in albumin promoter-B complexes within HuH-7 hepatoma cells incubated with 50 g/L albumin or dextran. The mRNA for synthesis of HNF1 is similarly decreased. The proposal of these authors is that COP acts homeostatically to regulate production of HNF1, which in turn controls transcription of serum albumin and several other liver-specific proteins.

d. Summary of Transcription Control Factors. To recapitulate the evidence for factors regulating transcription of the albumin gene, the anabolic hormones, insulin and growth hormone, are the ones most consistently involved in the positive sense. As yet no functional *cis*-acting site where they attach has been recognized. Growth factors HGF and EGF have minor stimulatory effects. Corticoids have supportive effects that are complex, time dependent, and related to

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actions of other regulators. Repression is seen with the acute-phase modulator, IL-6, and with the tumor-related factor, TNFα. The best candidate for action at a known site is HNF1, which may itself be regulated in some fashion by a colloid osmotic pressure transducer.

C. Translation

1. Translation Machinery

The albumin chain is assembled by the mechanism common to eukaryotes for the synthesis of proteins. Figure 5-2 shows the process in encapsulated form. A mature mRNA entering the cytoplasm through a nuclear pore is bound by ribosomes, dense snowman-shaped 200-Å bodies named for their high content of RNA. Here the mRNA acts in ticker-tape fashion to select the correct amino acids in the order dictated by its codons, proceeding from the NH_2 toward the COOH terminus of the protein. The components of the assembly mechanism are known in such detail as to be beyond the scope of this volume, but are readily available in reviews (Hershey, 1991).

a. General Mechanism. The mRNA is escorted by one or more "poly(A) mRNA-binding proteins," which bind tightly to the poly(A) tail and appear to enhance the efficiency of the translation process. Translation consists of three subprocesses: *initiation, elongation, and termination. Initiation* begins with the recognition of the Cap site at the 5' end of the mRNA by a 40S ribosomal subfragment (Altmann and Trachsel, 1993). With the aid of 10 or more **eukaryotic initiation factors (eIFs)** and energy from hydrolysis of ATP and GTP pyrophosphate bonds, the larger, 60S, ribosomal subfragment binds to form the 80S ribosome (Fig. 5-2). An activated form of methionine linked by its carboxyl group to its mRNA, Met-$tRNA^{Met}$, is already present on the complex. $tRNA^{Met}$ displays an anticodon for methionine, CGA, which pairs with the initiation codon, UAG, in the case of albumin 39 bp farther downstream on the mRNA from the Cap site. Chain assembly is now ready to begin.

Elongation proceeds in a cyclic manner. The tRNA bearing the anticodon for the next amino acid in line, with its amino acid attached (Lys-$tRNA^{Lys}$ in the case of HSA), binds to the next codon on the mRNA (AAG in HSA) at a grooved site **A** (amino acid) on the ribosome. The methionine residue, residing at an adjacent site **P** (peptide) is then shifted to the **A** site and its carbonyl group is moved from coupling with the tRNA to couple with the α-NH_2 group of the next amino acid (lysine for HSA). The first peptide bond has now been formed, and the Met-Lys-$tRNA^{Lys}$ complex is shifted *back* to the **P** groove.

Each subsequent addition occurs by the same steps. The charged RNA directed by the next codon (tryptophan in HSA) binds to the **A** site, the entire peptide shifts from the **P** to form the peptide bond, and the now-larger peptide

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(Met-Lys-Trp-tRNA^{Trp}) shifts back to the **P** site. For each added amino acid residue four or more pyrophosphate bonds are broken, one GTP each for the peptide shifts and two ATPs to form the aa-tRNA. Several **elongation factors** (**EFs**) participate in this process; the factor named EF-Tu is particularly important as a monitor of the accuracy of selection of the proper tRNA for the codon (Hughes and Tubulekas, 1993).

For each of the 61 codons that direct placement of amino acids there is a separate tRNA; the tRNA contains a site that recognizes the activating enzyme for that particular amino acid and an anticodon that can pair with one of the codons for that amino acid. Fortuitously, or perhaps by design, the abundance of the specific tRNA species parallels the codon usage frequency (Table 4-1) for a cell (Kurland, 1993). Free amino acids in the cytosol arrive either from the circulating pool in the plasma or from protein degradation in lysosomes and the cytosol itself. In tracer labeling experiments the specific activity of amino acids linked to their tRNAs is thus intermediate between the specific activity of those in the two pools.

Termination is directed by one of two possible terminator codons at the end of the translated region of the mRNA (UAA in exon 14 in HSA; the other codon is UGA). When this codon reaches the **A** site of the ribosome, a *release factor* present on the ribosome cleaves the aa-tRNA bond (the C-terminal leucine in HSA). The peptide chain does not return to the **P** site but is now free from the ribosome.

The rate of elongation has been found to be three to six cycles/s at 37°, *i.e.*, three to six amino acids are added to the growing chain each second. It is slower in cold-blooded animals, and is reduced by one-third by thyroidectomy in mammals (Haschemeyer, 1976).

More than one ribosome can work on an mRNA at the same time. About 6 s after initiation, when the Cap site of the mRNA now protrudes by about 36 codons from the complex, a second ribosome can recognize it, bind, and begin translation of a second protein molecule. This process, too, repeats, so that the whole translatable length of the mRNA becomes occupied with ribosomes. This large aggregate of ribosomes all at work on the same mRNA (Fig. 5-2) is termed a polyribosome or, more simply, a **polysome.**

b. Aspects Pertinent to Albumin. Only about 2% of mature albumin mRNA (detectable by its activity) is found in the nucleus (Ilio and Tamaoki, 1976). This mature mRNA for rat albumin has been observed to migrate from the nucleus in a cell-free system (Moffett and Webb, 1983). Its size is 17–19S (Taylor and Tse, 1976; Strair *et al.*, 1978), which would correspond to its calculated mass of 730 kDa [2256 bp including ≈200 bp in the poly(A) tail]; the observed mass was 770 kDa (Sala-Trepat *et al.*, 1979). Albumin mRNA appears to associate with one poly(A)-binding protein in particular (Johnson and Ilan, 1985).

Nascent (growing) albumin chains have been identified on isolated rat liver polysomes through the affinity of the more mature forms for antialbumin antibodies

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(Warren and Peters, 1965). Nascent albumin separated by immune precipitation (Jungblut, 1963b) or by its chromatographic behavior (Von der Decken, 1963) was one of the first proteins in which the N → C direction of chain growth was demonstrated, by showing that the C-terminal residues were more radioactive in short experiments; in early ribosome preparations only the completion of already-initiated chains occurred. A subsequent study by Sargent and Campbell (1965) confirmed the N → C mechanism with some of the earliest peptide patterns ("fingerprints") of rat albumin.

The time for completion of an albumin chain (608 residues in the rat) has been estimated as 1.5 min *in vivo* (Peters, 1962b), 2–2.5 min in the perfused liver (Jungblut, 1963b), and 2.3 min in isolated liver cells (Feldhoff *et al.*, 1977). The last figure is the average time calculated from the reported $t_{1/2}$ of 1.6 min,

$$t_{avg} = t_{1/2}/\ln 2. \tag{7}$$

A 2-min synthesis time corresponds to 5.0 residues/s, well within the range of 4–6 found for other proteins.

Polysomes synthesizing albumin have been isolated from rat liver cytoplasm that had been freed of membranes with detergents. More than 90% of the cytoplasmic albumin mRNA is associated with such polysomes (Iio and Tamaoki, 1976). They are large, corresponding in size to about 19 ribosomes per albumin mRNA; this density of one ribosome about every 34 codons is near the maximum possible and shows the albumin mRNA to be highly efficient for translation.

Estimation of the number of $mRNA_{SA}$ molecules per adult rat liver cell confirms this efficiency. The number determined chemically by hybridization was reported as about **3500**/cell by Muglia and Locker (1984); calculation from the figure of 7.3 μg mRNA/g liver (Liao *et al.*, 1986), assuming 200×10^6 hepatocytes/g liver and using the molecular mass for albumin mRNA of 770 kDa, gives **2852** mRNA molecules/cell.

The number of molecules of RSA formed per minute in an adult rat liver cell, calculating from the figure of 0.35 mg/g liver/h (Table 5-2), is 26,554; dividing by 19, the number of ribosomes working on a messenger simultaneously in a polysome, and multiplying by 2, the number of minutes needed to form one RSA molecule, gives **2795** polysomes or mRNA molecules/cell. This close agreement of synthetic activity with the observed number of 2850–3500 mRNA molecules present indicates that the mRNA molecules must nearly all be at work making albumin.

2. *Vectoring Growing Chain*

a. General Mechanism. Proteins destined for secretion follow a particular course even while they are being assembled. Such proteins contain as their first 18–20 amino acid residues a **signal peptide** (SP; Fig. 5-2) having a strongly

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TABLE 5-2
Relation of Albumin in Transit and Transit Time to Albumin Synthesis Rate[a]

Condition	Synthesis rate (mg/g liver/h)	Amount in microsomes (μg/g liver)	Minimum transit time (min)
Adult (1 year)	0.35	304	16.3
200 g (6–8 weeks)	0.73	389	16.0
Night	0.81	436	—
Fasted 24 h	0.55	346	14.8
Protein deficient	0.30	233	15
Protein deficient, refed 24 h	1.12	445	—
High-protein diet	0.51	322	15.6
Experimental nephrosis	0.77	341	14.4

[a]Data from Morgan and Peters (1971a) and Peters and Peters (1972).

hydrophobic midsection, which directs the growing chain through the lipid–protein membrane of the endoplasmic reticulum (ER) into the vesicular lumen. Here they are segregated from proteins designated for the cytosol (e.g., hemoglobin) or other organelles such as mitochondria. For the signal hypothesis, now amply proved, we are indebted to Günter Blobel of the Rockefeller Institute (Blobel and Dobberstein, 1975).

Much of the mechanism of this recognition and targeting has been revealed (Lingappa, 1989; Rapoport, 1992). A 54-kDa **signal recognition particle, SRP** (Fig. 5-2), acts as an intermediary by binding the signal peptide by its midsection in a hydrophobic pocket. Then, with the nascent chain, ribosome, and mRNA attached, it binds to a docking receptor on the ER membrane; at least six components of this receptor site have been distinguished. ER to which polysomes are attached is termed **rough ER** (RER), from its studded appearance, in contrast to **smooth ER,** which has no such attachments.

When the growing chain has reached 50–75 residues, the SRP is removed and the chain is transferred into the membrane; the ribosome simultaneously becomes attached to the membrane surface at a ribosome receptor (Wolin and Walter, 1993). Details of the translocation of the nascent chain are unclear, but it appears that the chain is protected from the cytosol in a hydrophilic tunnel in the ribosome and that the signal peptide binds in a looped configuration in an aqueous pore in the membrane (Shaw *et al.*, 1988; Crowley *et al.*, 1993).

By the time the chain reaches the lumen of the ER its signal peptide has apparently already been cleaved (Fig. 5-2). A **signal peptidase,** located in the

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membrane at the translocation site, recognizes the trailing end of the signal peptide by its more hydrophilic nature, as compared to the midsection. The cleavage site is usually the COOH side of a small amino acid (Gly, Ser, Ala, Cys) preceded in the –3 position by another such residue, but other nearby residues and "intrinsic structural features" of the peptide can influence the fidelity of cleavage (Nothwehr and Gordon, 1990).

b. Aspects Pertinent to Albumin. The signal peptide sequence for (rat) albumin was first reported by Strauss *et al.* (1977); it and those known for other albumins appear in Fig. 4-3. The signal peptide for chicken albumin, MKWVTLISFIFLFSSATS (Compere *et al.*, 1981), is highly homologous. In amphibians and higher order animals the signal peptide contains 18 residues; its length is uncertain in the salmon, and is longer, 23 residues, in the lamprey. The cleavage site almost invariably lies after a Ser residue, which is part of an SSAYS- sequence in six of the seven mammalian species displayed. Cleavage of the rat albumin signal has been observed *in vitro* by ascites cell membranes (Strauss *et al.*, 1978). In humans, the –2 R → C mutation in Albumin Malmö-1 (Redhill) (Table 4-8) is proposed to have created an alternate signal peptide cleavage site that results in Arg-albumin appearing as 30% of the circulating albumin.

It was early realized that essentially all intracellular albumin is segregated within ER vesicles (Gordon and Humphrey, 1961; Peters, 1962a); a small portion is within larger organelles that sediment with the mitochondria, apparently albumin undergoing degradation within lysosomes (see Section III). The demonstrations that the earliest incorporation of labeled amino acids into albumin occurs in the microsome fraction and that isolated microsomes can form albumin led to studies of the extractability of this albumin and the nature of its association (Jungblut *et al.*, 1959; Peters, 1959a), reviewed by Campbell (1960). Generally it was found that the newly formed albumin became highly soluble on dissolving the lipid of the membrane with detergents such as deoxycholate or Triton; significant amounts could be released merely by freeze–thaw cycles or carbonate at pH 9.

When albumin-forming polysomes were isolated or identified by complexing with either antialbumin antibodies (Takagi and Ogata, 1971) or Fab fragments (Ikehara and Pitot, 1973), nearly all, 90% or more, were found to be membrane associated in the form of rough ER. Of albumin mRNA, detected by hybridization with a cDNA probe, 98% was with membrane-bound polysomes and only 2% was with free polysomes (Yap *et al.*, 1977).

3. Control of Rate of Translation

As with any assembly process, translation needs raw materials, energy, and the assembly machinery, and will slow or stop in the absence of any of these.

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For albumin the raw materials are amino acids, energy is pyrophosphate compounds, and the machinery is the complex ribosomal mRNA structure just described.

Any protein requires amino acids for assembly, but albumin synthesis has long been known to be particularly sensitive to protein depletion, albumin levels falling much more severely than do globulin levels when dietary protein is restricted (Weech *et al.*, 1935). Protein-free diets (but containing ample carbohydrate) lower the rate of albumin synthesis in rats by 70% *in vivo* (Morgan and Peters, 1971a) and by 80% in liver slices (Peters, 1973). Albumin synthesis cannot respond to demands for greater production during nephrosis or hypoalbuminemia unless a high-protein diet is provided (Kaysen *et al.*, 1989).

Total starvation over a period of 1–3 days, oddly, does not depress the albumin synthesis rate as much as does omission of protein from the diet. In this case the rate drops by 33–40% *in vivo* (Peters and Peters, 1972), 65% in the perfused rat liver (Rothschild *et al.*, 1968), and 60% in liver slices (Peters, 1973). This should not seem so surprising, however, when it is considered that in fasting, once the glycogen stores are gone, the body turns to degrading its own tissue proteins for energy; the resulting amino acids maintain the plasma free amino acid levels near normal (Yap and Hafkenscheid, 1981) whereas in protein depletion these levels fall.

Even the lack of a single essential amino acid, one of the 10 or so species that cannot be formed by the liver, can cause albumin formation to slow. If a normal rat liver is perfused with blood from protein-deficient rats, albumin synthesis falls 40%, but is restored to normal by adding the three branched-chain amino acids, leucine, isoleucine, and valine, which are the ones most severely lacking in the plasma during protein depletion (Kirsch *et al.*, 1969).

The effects of amino acid deficiency are short-lived, albumin synthesis being restored fully after a 6-h meal infusion in man (De Feo *et al.*, 1992) and even more rapidly after giving a "meal" in the form of free amino acids by stomach tube in rats (Morgan and Peters, 1971a). Synthesis measured *in vivo* rose 50% within 1 h and to normal by 4 h; increased albumin within the liver was detected within 15 min.

Mere lack of amino acids as building blocks cannot explain why albumin biosynthesis is so particularly dependent on their presence, because all proteins require amino acids. Protein depletion and starvation have been known to affect elements of the assembly apparatus in diverse ways. Polysomes detach from ER membranes and appear unbound in the cytosol (Everson *et al.*, 1989); albumin mRNA detaches from the polysome complexes and is found linked to proteins as 30–50S particles in the cytosol (Yap *et al.*, 1978). Addition of certain amino acids to the perfused liver from a fasted rabbit causes the polysomes to reaggregate and albumin synthesis to resume (Rothschild *et al.*, 1974). Two amino acids that were particularly effective are tryptophan and ornithine;

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ornithine apparently acts to increase production of the polyamine, spermidine, which enhances mRNA–ribosome binding through a lysine to "hypusine," N^{ϵ}-(4-amino-2-hydroxybutyl)-L-lysine (Park *et al.*, 1993).

Amino acid deprivation can affect control points in the initiation process. Lack of a single amino acid such as histidine increases the phosphorylation of the initiation factor, eIF-2α, at its Ser-51 site (Kimball *et al.*, 1991). Presence of this phosphate group blocks the action of eIF-2 in binding of met-tRNA^{Met} to the 40S subunit (Fig. 5-2) and down-regulates protein synthesis. Phosphorylation of Ser-53 of factor eIF-4E, on the other hand, stimulates protein synthesis; this factor acts in the binding of the mRNA to the 40S subunit, and its phosphorylation is reported to be enhanced by insulin (Rhoads, 1993).

Many other components of the elaborate assembly mechanism may at times be rate limiting. Not only must the amino acid appropriate for the next codon in line be available in charged form, but it must be attached to the particular tRNA for that codon as well. Because the albumin mRNA codon usage (Table 4-1) is in many ways unique—favoring AGA for Arg, GGA for Gly, and TTT for Phe, for example—one can easily see how local shortages of charged tRNAs, even if brief, could delay assembly of the albumin chain.

Assuredly the most important component of the assembly apparatus in controlling the rate of protein synthesis is the mRNA level, or, more specifically, the level of "translatable" mRNA, i.e., that which is available for action on ribosomes. This level is the resultant of its synthesis by transcription, discussed in the preceding section, and its degradation or inactivation. The normal half-life of albumin mRNA in rat liver appears to be in the vicinity of 22 (Peavy *et al.*, 1985) to 26 h [calculated from the figure of 75% disappearance in 36 h after initiation of the acute-phase reaction (Schreiber *et al.*, 1986)]. Factors that speed mRNA degradation without altering its synthesis would decrease its level and slow the formation of albumin just as readily as would increased synthesis. A lack of free amino acids appears to be one of these factors.

As early as 1978 Pain *et al.* reported a 50% drop in translatable mRNA for albumin in rat liver during dietary protein deficiency. This drop is selective for albumin, and, like the albumin synthesis rate, is more marked when ample energy (carbohydrate) is still provided than during total fasting (Sakuma *et al.*, 1987; de Jong *et al.*, 1988). With hepatocyte cell cultures, omission of a single essential amino acid (Hutson *et al.*, 1987), particularly tryptophan or phenylalanine (Straus and Takemoto, 1988), was accompanied by a fall in mRNA concentration parallelling the fall in albumin synthesis rate.

In rats on a low-protein diet Straus and Takemoto (1990) showed that the observed 62% decrease in albumin mRNA concentration was unaccompanied by a decrease in the rate of albumin gene transcription, a finding that pointed the finger at increased degradation, i.e., shorter half-life, of the existing mRNA as the cause of the depressed synthesis of the protein. How an increased supply of

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amino acids affects mRNA stability is obscure; a hormonal action or some direct effect are two possibilities.

A decline in serum albumin mRNA occurs with estrogen administration in cockerels (Williams *et al.*, 1978) and in *Xenopus laevis*. Two laboratories have now shown that estrogen does not affect albumin mRNA formation, but accelerates its degradation (Kazmaier *et al.*, 1985; Schoenberg *et al.*, 1989). Although mRNA stability is considered to be directly related to the length of its poly(A) tail, *Xenopus* albumin mRNA has a very short tail, only 17 residues, so that Schoenberg *et al.* (1989) consider that the regulation by estrogen involves a unique pathway. A nuclease that selectively destroys albumin mRNA in *Xenopus* is under study (Pastori and Schoenberg, 1993).

D. Secretion: Proalbumin and Its Possible Roles

The cleavage product after removal of the signal peptide from albumin is *proalbumin*, named for a six-residue peptide sequence at its amino terminus called the *propeptide*. In most mammals the sequence is Arg-Gly-Val-Phe-Arg-Arg (see Fig. 4-3, residues –6 to –1); similar propeptides occur in *Xenopus* and lamprey albumins, but are not identifiable in salmon albumin, AFP, ALF, or DBP. The consensus sequence begins with a basic residue and ends with two basic residues, K/R-X-X-X-K/R-R. The middle three amino acids are hydrophobic.

Realizing that the albumin chain is formed quickly but is not secreted for 20–30 min, investigators sought to characterize the intracellular form. The added cationic amino acids reduce the negative charge of proalbumin to three units less than that of albumin, so the electrophoretic mobility of proalbumin is slightly less than that of mature albumin (Fig. 4-7). The intracellular precursor form was isolated in early studies, but the crude electrophoretic techniques of that day (using paper or agar as a supporting medium) did not permit detection of a difference in migration (Peters, 1957). Jungblut *et al.* (1959) did find small amounts of a more slowly migrating albumin antigen on immunoelectrophoresis in agar; techniques for identifying amino-terminal arginine residues were so poorly developed, however, that the propeptide sequence was missed in another study (Peters, 1959a) (or it had been artifactually cleaved during isolation).

Hill *et al.* in 1972 and Tamaoki *et al.* in 1974 noted that the intracellular form of newly synthesized rat albumin migrated more slowly than circulating albumin. But it was nearly 15 years after Jungblut's work before others identified a slower moving albumin form on isoelectric focusing or ion-exchange chromatography (Judah *et al.*, 1973; Russell and Geller, 1973), which could be converted to albumin by mild trypsin treatment. Edman degradation gave first a sequence of GVFSR (Urban *et al.*, 1974) and then the correct sequence of RGVFRR (Russell and Geller, 1975) for the added oligopeptide.

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Propeptides are not unique to albumin but occur in many secreted proteins (Peters, 1987). Several of these (insulin, haptoglobin) are situated internally in the peptide chain and are required for proper alignment of Cys groups prior to S–S bond formation; others protect an enzyme from causing premature damage (trypsinogen). Leader propeptides occur in apolipoprotein A-II, fibronectin, and several clotting proteins; the leader peptide of proparathormone, Lys-Ser-Val-Lys-Lys-Arg, resembles that of proalbumin and was discovered about the same time (Hamilton *et al.*, 1974). The function of leader propeptides is not certain; options are discussed below (Section I,D,4).

By convention precursor protein forms carrying a signal peptide are termed *pre*proteins; those carrying a propeptide are termed *pro*proteins; those carrying both are termed *prepro*proteins. Thus, the initial translation product of albumin is preproalbumin, and its chief intracellular form is proalbumin. Confusion arises when referring to the plasma protein, *prealbumin*, or *transthyretin;* it was originally named for its more rapid electrophoretic migration, compared to albumin, on electrophoresis at pH 8.6. Because the prefix, *pre-*, generally denotes a metabolic precursor, and prealbumin (transthyretin) bears no genetic or metabolic relationship to serum albumin, the term *transthyretin* was introduced as a more accurate term than *prealbumin*.

1. Pathway of Secreted Proteins

The general pathway followed by proteins vectored to the ER by virtue of their signal peptides and destined for secretion has been elaborated chiefly by G.E. Palade and co-workers at Rockefeller and Yale universities (Palade, 1975). They studied the products of the exocrine pancreas and showed that the newly formed proteins move, always within vesicular channels, from rough ER via smooth ER to the *cis-* (central) plates of the Golgi complex, then through the Golgi apparatus to its *trans-* (peripheral) side before leaving the cell by exocytosis (Fig. 5-4). Numerous reports have documented that plasma proteins move through this pathway as well.

It is at the *trans*-Golgi face that the pathway of secretory and hepatic proteins splits. Albumin and other plasma proteins are found in large vesicular carriers, whereas proteins destined to reside in the hepatocyte membrane are in small smooth Golgi vesicles (Saucan and Palade, 1994). Several recent reviews are of value (Lingappa, 1989; Pelham, 1989; Halban and Irminger, 1994).

The nascent proteins begin to form their disulfide bonds and attain native configuration even before translation is complete (Bergman and Kuehl, 1979). Chaperone proteins, collectively called *chaperonins*, bind to incompletely folded proteins and shield them in a protected grasp while they complete this process (Ellis and van der Vies, 1991). Addition of carbohydrate chains in the case

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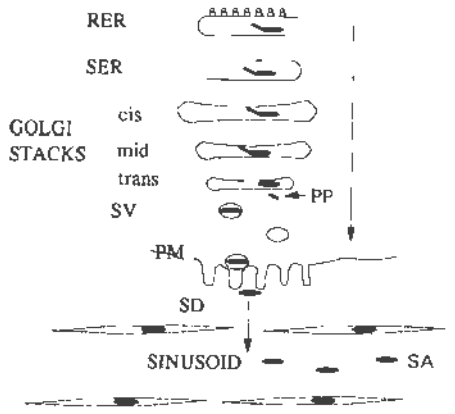


Fig. 5-4. Schematic model of secretion pathway of albumin in the hepatocyte. RER, Rough endoplasmic reticulum; SER, smooth endoplasmic reticulum; PP, propeptide; PM, plasma membrane; SD, space of Disse; Sv, secretory vesicle.

of glycoproteins may also begin before the peptide chain is complete, *i.e.*, cotranslationally.

Details of the mechanism whereby vesicles hook to one another and then transship their contents along this pathway have only recently appeared, and have been reviewed by Rothman and Orci (1992). The mechanism involves acylation by hydrophobic prenyl fatty acids and interaction of "SNAP" attachment proteins and "SNARE" receptors (Söllner *et al.*, 1993); it is interesting reading but regrettably is beyond the scope of this volume.

2. *Disulfide Bond Formation by Albumin*

The ability of fully reduced albumin to reform its native disulfide-bonded structure *in vitro* has been noted (Chapter 2, Section II,C,3,b); albumin appears to be the first protein whose disulfide bonding was studied *in vivo*. Peters and Davidson (1982) showed that infusion of iodoacetamide into the rat portal vein was an effective means of "freezing" thiol groups of proteins *in vivo* before the formation of disulfide bonds. They found very little remaining thiol on completed proalbumin within the rough ER, only an average of one SH more than the persistent thiol at CySH-34; this additional SH was shown to lie in the C-terminal domain. The remaining thiol, even of nascent chains, appeared to average only about 45% of their total Cys/2 groups.

Their results agree with a picture in which, like the immunoglobulin light chains studied by Bergman and Kuehl (1979), disulfide bonding begins even before the chain is completed, when it is about 200 residues long, and proceeds in an N → C direction. All of the 17 disulfide bonds of albumin are completed within 0.5 min of release of the completed chain from the parent polysome.

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Why the first thiol in the chain, at CySH-34, does not participate in S–S bonding is not clear. It is not necessary for reforming S–S bonds *in vitro* (Chapter 2, Section II,C,3,b). Possibilities are that it is held closely to the negative membrane by the positively charged propeptide, or that its distance from the S–S bonds in the tertiary conformation of albumin precludes its bonding.

Such a simple mechanism is possible in the case of albumin and light chain because their S–S bonds occur sequentially along the peptide chain, without overlap (Fig. 2-1). Hence they are free to pair shortly after emerging into the cisternal space of the rough ER. Chaperonins may participate in folding and S–S bond formation of albumin, but their action has not yet been detected. Generally only proteins with more complex S–S bonding patterns, with overlap from remote regions of the chain and which may form "wrong" disulfide bonds, require the protection of a chaperone protein and a longer time to complete folding and S–S bridging.

The reaction mechanism for the oxidation of two SH groups to form an S–S group is obscure. *In vitro* the presence of glutathione is helpful, probably through the formation of mixed disulfides:

$$\text{Alb-SH-SH} + \text{GSSG} \rightarrow \text{Alb-SH-SG} + \text{GSH}; \quad (8)$$

$$\text{Alb-SH-SG} + \text{GSSG} \rightarrow \text{Alb-S–S} + \text{GSH}. \quad (9)$$

This mechanism may operate in the hepatocyte microsomes; the intermediate mixed disulfide forms have not been detected, but their existence could be very brief.

Several enzymes intrinsic to the wall of the rough ER contribute to protein folding. Protein disulfide-isomerase (Chapter 2, Section II,C,3,b) accelerates folding *in vitro* and may be important in the reshuffling of mismatched S–S bonds, but should not be essential for albumin folding. An NADP-linked glutathione oxidase is a suggested means of disposing of the hydrogen atoms removed from the cysteines and passing them along to the oxidative pathway (Ziegler, 1985). Finally, a proline isomerase acts to convert a portion of the energetically favored *trans*-proline bonds to *cis*-bonds. Only about 7% of X-Pro bonds are in the *cis* form in proteins of known tertiary structure (Schmid *et al.*, 1993), but the isomerization is slow without enzymatic assistance. The proportion or location of *cis*-proline bonds in albumin is not as yet known.

3. Secretory Pathway of Albumin

Albumin follows the same path as other plasma proteins through the hepatocyte cytoplasm (Fig. 5-4). Awareness of this pathway developed as cytochemical techniques improved; first only whole microsomes could be isolated (Peters, 1957), then rough- and smooth-surfaced vesicles (Peters, 1962a; Jungblut,

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1963a), and then the Golgi apparatus (Fig. 5-5). From the Golgi complex the new albumin appears to travel in smooth secretory vesicles to the cell membrane at the sinusoidal face of the cell. During its transit newly formed albumin is always contained within vesicles; it is bound only loosely if at all to the walls, being one of the most easily extracted secretory proteins (Redman and Cherian, 1972), in contrast to glycoproteins, which require more extensive processing. Movement along the pathway is sequential, with little evidence of pooling except perhaps in the final secretory vesicles. By this stage some broadening is seen in the time curve of labeled albumin after a short pulse of [^{14}C]leucine (Morgan and Peters, 1971a).

There are no storage or collecting vesicles for albumin, and there is no evidence that albumin secretion proceeds on demand.

Within the Golgi complex albumin has been seen by immunohistochemical microscopy in stacked cisternae including their peripheral dilatations (Yokota and Fahimi, 1981). The distribution varied among different Golgi bodies—albumin was usually present in the *trans* stacks, but was absent in some of the *cis* and middle regions. One tracer study has found evidence suggesting that albumin bypasses the central cisternae in favor of peripheral Golgi elements en route to the *trans* face (Franz *et al.*, 1981).

Albumin shares the same vesicle with other plasma proteins in the late stages of its travels through the liver cell, whereas proteins destined to become membrane constituents use other vesicular pathways (Saucan and Palade, 1994). Albumin, transferrin, and vesicular stomatitis virus glycoprotein are found in

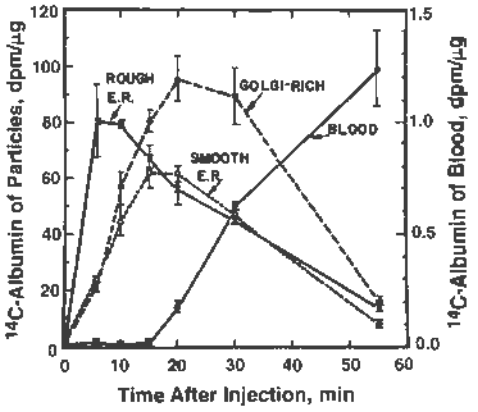


Fig. 5-5. Location of [^{14}C] leucine-labeled rat albumin within rat liver cell organelles as a function of time after injection of a tracer dose of leucine by tail vein. Reprinted from Peters *et al.* (1971) by permission of The American Society for Biochemistry & Molecular Biology

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dilated cisternae, apparently derived from *trans*-Golgi elements (Strous *et al.*, 1983). Albumin and lipoprotein are also intermixed in vacuoles of the *trans* face (Yokota and Fahimi, 1981). Prior to this point, in the rough ER and in *cis*-Golgi elements, the two proteins appear to travel in separate vesicles.

Release of albumin from a secretory vesicle through the cell membrane is assumed to involve the membrane fusion processes described above, with the vesicle blending into the cell membrane and its contents then discharging passively into the space of Disse between the hepatocyte and the sinusoidal wall (Fig. 5-4). Microtubules appear to move the vesicles to the sinusoidal face, because the presence of colchicine, an inhibitor of microtubule assembly, shuts off 90% of the albumin release into the plasma (Redman *et al.*, 1978). A very small amount, about 0.3%, of the newly formed albumin is secreted into the bile. This mechanism does not utilize microtubules, because colchicine *increases* the secretion fivefold (Saucan and Palade, 1992). Perhaps the biliary secretion is merely leakage, which increases with colchicine due to the backing up of unsecreted albumin.

4. *Conversion of Proalbumin to Albumin*

Cleavage of the propeptide of proalbumin is one of the last events before mature albumin is released from the liver cell, occurring either in the *trans*-Golgi compartment or in the secretory vesicles (Fig. 5-4) (Ikehara *et al.*, 1976). The cleavage enzyme, or convertase, has only recently been identified. Trypsin in extremely low concentrations (1 μg/mL) will cleave proalbumin readily, not surprising considering the double Arg-Arg sequence at the point of cleavage. But trypsin is active, like other serine proteases ("serpins"), at mildly alkaline pH, near 9, and the vesicles in which proalbumin is cleaved appear to be mildly acidic.

The catalytic domain of the responsible enzyme *in vivo* is related not to the serpins but to the bacterial enzyme, subtilisin. It was first found in yeast as the endoprotease, KEX2 (Brennan *et al.*, 1990c); the related protein in eukaryotes is termed *furin* because its gene sequence resembles that of the *fur* oncogene (Van de Ven *et al.*, 1991). Proalbumin conversion has now been induced in COS-1 kidney cells by transfection with rat cDNA for furin (Misumi *et al.*, 1991). Cleavage activity *in vitro* requires calcium ions and is optimal at pH 6. It is inhibited by Hg^{2+}, but not by the usual serpin inhibitors such as sulfonyl fluoride or chloromethyl ketone or by pepstatin (Brennan and Peach, 1991).

Furin, or perhaps furins, are held in the membrane of *trans*-Golgi elements and secretory vesicles by a hydrophobic transmembrane peptide segment. Proforms of proteins cleaved in secretory vesicles generally have terminal paired dibasic residues such as the leader peptides of proalbumin and proparathormone,

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or even the internal C-peptide of proinsulin. The furin in the *trans*-Golgi membrane has a higher specificity, cleaving proforms such as the vitamin K-dependent coagulation factors (RPRR) and the proinsulin receptor (RKRR) (Barr, 1991; Steiner *et al.*, 1992).

Chicken proalbumin, which has the unusual propeptide RNLQRFAR (Hachè *et al.*, 1983) with but a monobasic terminal site, is also cleaved by the rat liver convertase; the Arg residue at position –4 appears to be critical (Brennan and Peach, 1991). Among the variant human proalbumins (Table 4-8 and Fig. 4-7), there is little or no processing of the –2 Arg → His, –1 Arg → Gln, –1 Arg → Pro, or –1 Arg → Leu mutants. The –2 Arg → Cys form is cleaved at about 25% of the rate of human proalbumin (Rennan *et al.*, 1990c); the CySH residue can apparently satisfy the requirements of the enzyme.

The residue at the +1 position also affects the cleavage. Neither Albumin Blenheim, 1 Asp → Val, nor rat proalbumin modified by site-directed mutagenesis to replace its 1 Glu with Val, Leu, or Ile, is processed (Oda *et al.*, 1991; Brennan and Nakayama, 1994). A 1 Glu → Ser substitution is acceptable: apparently only a hydrophobic residue cannot be tolerated.

The variant proalbumins that cannot be processed to albumin appear in the circulation in a concentration equal to that of albumin (about 23 g/L each), and survive for the normal life span of the albumin molecule. *Normal* proalbumin is undetectable in blood, but when it is injected intravenously into a rat it is converted to albumin at a rate of about 5% per h, apparently on passage through the liver (Peters and Davidson, 1984). Normal human proalbumin has been reported in blood plasma in one instance of hepatitis with superinfection by the δ particle (Fine *et al.*, 1986), in another complicated by hepatic metastatic disease (Cesati, 1993), and in two instances in children with the α_1-antitrypsin mutation, 358 Met → Arg, which causes this protease inhibitor also to inhibit the proalbumin convertase, furin (Brennan *et al.*, 1993). In the latter two cases proalbumin made up only 3–5% of the total serum albumin; this steady-state level is the resultant of secretion and conversion in the circulation. The albumin secreted by fetal mouse liver prior to 16 days of development is in the form of proalbumin; the converting mechanism for processing apparently develops only slowly (Hannah *et al.*, 1980).

Free propeptide has not been detected in liver or blood (<1 μM). When synthetic propeptide, labeled by tritiation of an iodotyrosine form, is injected intravenously into a rat it disappears from the blood within 2 min (Peters and Davidson, 1986). If it is incubated with rat liver cell fractions it is rapidly degraded; smooth membrane fractions are especially active in removing the terminal Arg residues, while the cytosol cleaves the resulting oligopeptides to their constituent free amino acids. Oda *et al.* (1990) have prepared a propeptide-directed antibody, but they do not appear to have applied it to the immunochemical determination of proalbumin or propeptide at microlevels.

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What is the role of the propeptide? The free form appears to be too scarce and too transient to act as a negative-feedback regulator of albumin synthesis. Proalbumin, isolated in a 25-mg amount from rat liver (Peters and Reed, 1980), contained no carbohydrate groups that might have signified transient glycosylation, and it refolded as well as but no better than did mature albumin after complete reduction. It showed similar helical structure when tested by CD (Schreiber and Urban, 1978), and bound palmitate and bilirubin equally as well as did albumin. Hence the additional peptide does not block a binding site in the manner that an amino-terminal peptide blocks the powerful potential enzymatic action on trypsinogen. Nor could any difference in affinity for cytoplasmic membranes be detected.

When the gene for rat albumin was genetically engineered to remove the propeptide and then inserted into COS cells, the signal peptide was cleaved normally, but the resulting rat albumin was secreted at a rate much slower than normal, and albumin accumulated in the ER (McCracken and Kruse, 1989). Hence the propeptide appears to promote the desired migration of proalbumin; a similar conclusion was reached for the propeptide of human apolipoprotein AI (McLeod *et al.*, 1990). Although enhancement of binding of proalbumin to cytoplasmic membranes could not be demonstrated *in vitro*, cationic charges are known to affect membrane attachment by proteins, and it would seem that the leading propeptide in some way acts as a pilot to guide proalbumin from its source in the rough ER to the secretory vesicles for secretion.

5. *Kinetic Aspects of Albumin Secretion*

Inhibitors can block the synthesis and secretion of albumin at various points (Redman and Banerjee, 1983). Puromycin causes premature release of nascent albumin from polysomes, whereas cycloheximide inhibits its release. In either case albumin synthesis is quickly stopped. Blocking ATP production with dinitrophenol or anoxia inhibits transfer from the rough ER to the Golgi complex. Monensin and colchicine cause albumin and other secretory proteins to accumulate in the Golgi vesicles. Many protease inhibitors stop albumin secretion (Algranati and Sabatini, 1979); they do not block the proalbumin convertase because albumin and not proalbumin is still slowly secreted, and they may inhibit some other essential proteolytic step.

Cycloheximide has been a useful tool with which to examine albumin secretion. Given to an intact rat, it stops incorporation of a tracer amino acid into nascent albumin within 1–2 min (Glaumann, 1970; Peters and Peters, 1972). The movement of albumin through its secretory pathway is unaffected, however, and the delivery of newly formed albumin to the blood is not retarded. Figure 5-6 shows how the albumin within microsomes (rough ER, smooth ER, and the Golgi complex) continues to drain at a steady rate from the hepatocyte after cycloheximide is given. The rate is the same as that before the inhibition of albu-

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min synthesis, higher in young nephrotic rats than in adult rats. These studies show that the secretory process whereby newly formed albumin moves through the organelles of the cytoplasm is not dependent on a continued supply of albumin from the polysomes, but that the presence of albumin within the vesicles is sufficient inducement to its processing and secretion.

The amount of albumin found within the secretory pathway is just under 400 μg per g liver in a young (8-week-old) rat. If the rate of synthesis rises, the channels distend with more albumin; the total content rises and falls with the rate of synthesis in a direct manner (Table 5-2 and Fig. 5-7). This buildup is further evidence that secretion is independent of synthesis, and merely responds to the presence of albumin in the vesicular channels. Unlike hormones such as insulin or parathormone, which are concentrated and stored in condensing vacuoles and released in response to signals such as blood sugar or calcium levels, the release of albumin occurs at the end of its passage through the hepatic cytoplasm and there is no significant amount of albumin in storage.

The time required for proteins to be secreted, mentioned in Section I,A, has been measured under a variety of conditions. Green and Anker (1955) tested the effect of temperature on the lag in appearance of labeled glycine or leucine in plasma proteins in several animals. This "transit time" rose from about 25 min in rabbits at 37° to 2.5 h at 25°, and from about 1 h in turtles at 30° to 5.5 h at 10°. Haschemeyer (1973) reported a similar albumin lag time in the toadfish at 10°

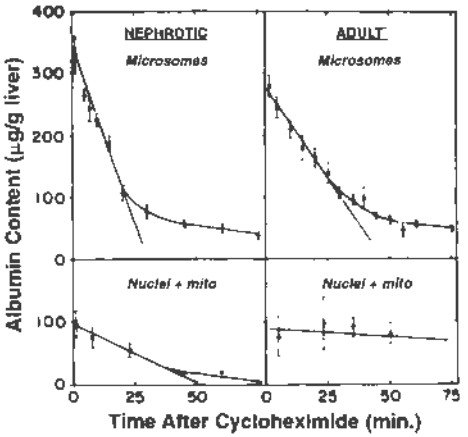


Fig. 5-6. Content of albumin, determined immunochemically, in rat liver organelle fractions as a function of time after inhibiting albumin synthesis with cycloheximide. The albumin within "nuclei + mito" is believed to lie largely within lysosomes. Reprinted from Peters and Peters (1972) by permission of The American Society for Biochemistry & Molecular Biology.

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and about 1 h at 20°, and Fries and Lindstrom (1986) found about 1 h for cultured rat hepatocytes at 20°.

More precision can be obtained with shorter pulses of the tracer amino acid, which were made possible as the specific radioactivity of the available labeled compounds rose, so that experiments could be conducted with much smaller doses of the tracer. That is, the administered amino acid did not raise the level of the amino acid in the blood or the liver significantly and acted more nearly as a true tracer of metabolism. The transit times for albumin in rats thus obtained (Table 5-2) show a consistent *minimum* lag time of 14–16 min for appearance of isotope in secreted albumin after injection in the tail vein (Fig. 5-5). The *average* time is about 25 min; this can be considered as the sum of a 15-min period of movement followed by a pooling and random release from secretory vesicles with a $t_{1/2}$ of $\approx$7 min (Morgan and Peters, 1971b). The *maximum* time approaches 50 min.

The minimum transit time is not a function of secretion rate, but remains constant at 14–16 min over a threefold range of synthesis (Table 5-2); nor is it affected by occurrence of the acute-phase reaction following injury (Jamieson and Ashton, 1973; Myrset *et al.*, 1993). The situation is similar to a factory that responds to an increased demand for its product not by speeding up the assembly line, but by adding extra production lines.

Transit times for other plasma proteins are usually longer than that for albumin; minimal delays for albumin, α_1-antitrypsin, and transferrin are 16, 23,

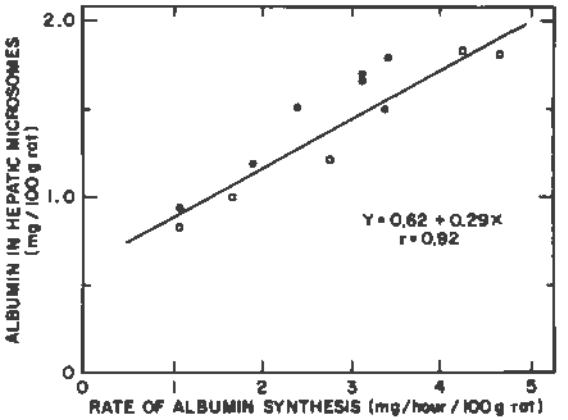


Fig. 5-7. Content of albumin, determined immunochemically, in rat liver microsomes as a function of the rate of albumin synthesis under different conditions. ●, data from Peters and Peters (1972); □, data from Morgan and Peters (1971a). Reprinted from Peters and Peters (1972) by permission of The American Society for Biochemistry & Molecular Biology.

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and 31 min, respectively, with proportionately longer maximal times for secretion (Morgan and Peters, 1985). Transthyretin and retinol-binding protein times were even longer (Fries *et al.*, 1984), whereas the time for prothrombin approximated that for α_1-antitrypsin (Kvalvaag *et al.*, 1988). For all of these plasma proteins the delays occurred mainly in the rough ER, whereas the half-time spent in release from the Golgi complex was 10–15 min; this can be likened to a slower assembly line for many proteins than for albumin, but a similar time in the shipping department. It has not been possible to correlate the time spent in the ER with processes such as glycosylation or γ-carboxylation, however. The heavily glycosylated α_1-acid glycoprotein (orosomucoid), for example, is secreted as rapidly as is carbohydrate-free albumin (Jamieson and Ashton, 1973).

E. Rate of Biosynthesis

In its roles as a model protein, as the major protein produced by the liver, and as an important index of good nutrition and health, there is increasing interest in measuring the rate of albumin biosynthesis, and considerable effort has been expended in devising methods for this purpose. This section discusses the approaches used, *in vitro* and *in vivo*, and lists the rates found for various conditions. [For further review see Peters (1983).]

1. Methods for Measuring Rate of Albumin Biosynthesis

a. Net Synthesis. The most straightforward measurement of the rate of biosynthesis is the mass of albumin put out by the liver in a given time. This is obviously impractical in the living animal; one requirement is that the surrounding medium be free of albumin of the host species so that the secreted albumin can be determined by an immunoassay. Slices and cultured cells have been used, but the departure from normal hepatic structure makes for poor production; note the low output for these systems in Table 5-1. They are useful for comparing effects of changing conditions, not for determining the inherent ability of the liver.

The perfused liver has been used more successfully. Rates 70–90% of those measured by other techniques *in vivo* have been obtained in rat livers perfused with human blood (Gordon and Humphrey, 1961) (Table 5-1). Care must be taken to distinguish synthesis from washout of existing extracellular albumin or continued secretion of intracellular albumin when synthesis has been inhibited, for instance, by the administration of cycloheximide described above. With the perfused liver (or with liver slices) a preincubation washout period of 1 h or more is needed.

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b. Incorporation of Tracer Amino Acids. Numerous approaches to measuring albumin synthesis by the rate of uptake of amino acids from a trace-labeled pool have appeared. The common problem is to detect the labeling of the immediate precursor pool—the intrahepatic cytosol pool or, better, the pool of the labeled amino acid charged to its tRNAs. If this specific activity can be maintained constant, it is an easy matter to measure the linear slope of its appearance in albumin. The alternative is to give a small dose of highly labeled tracer amino acid, which creates a "pulse" of about 5 min of label in the cytosolic pool, then follow this pulse of label into the labeled albumin, which appears 15–30 min later. *In vitro*, as with slices or cell cultures, the latter technique is usually called a "pulse-chase" experiment and is primarily useful in following secretory pathways.

The sharp pulse has been used successfully *in vivo* (Haider and Tarver, 1969) in rats but is not useful in humans. In one study, rat liver samples are obtained over the period 0–10 min after injection of L-[^{3}H]leucine to obtain the total amount of label in the cytosolic amino acid pool. The label in the precursor pool was obtained by integrating the specific activity of the cytosolic leucine over the 10 min that it was detectable. The label in albumin was captured either as that in microsomal albumin 16 min after the injection, a time when synthesis is complete and secretion from the cell has not begun, or as that in circulating albumin 2–4 h after injection, after all of the labeled albumin has been secreted; the methods gave comparable results even though the use of circulating labeled albumin requires a measurement of plasma volume and correction for a small amount of loss from the plasma during the time after secretion (Morgan and Peters, 1971a). A refinement not yet tested could be the isolation of the leucine-tRNA^{Leu} pool for specific activity determination (Wallyn *et al.*, 1974). Only the short-pulse method is useful to measure albumin synthesis within time periods between 16 min and a few hours.

Two techniques of labeling the precursor free amino acid pool at a near-constant level in man have been popular. The direct approach is either to flood the pool by one or more massive doses of the tracer, thus setting the specific activity as that found in the large plasma free amino acid pool, or to infuse a highly labeled tracer until the plasma amino acid reaches a constant specific activity. The second approach, administration of tracer until the plasma label becomes constant, requires 3–6 h in the rat and 10–30 h in man.

The first approach, flooding the system, has been applied to liver slices, cells, the perfused liver (Richmond *et al.*, 1963), and the whole animal (Ballmer *et al.*, 1990), but is unphysiological in raising the concentration of the amino acid injected *in vivo* or present in the incubation medium. Amino acids in large (greater than tracer-level) doses can have deleterious effects, such as the depression of leucine incorporation into tRNA and protein by phenylalanine (Roscoe *et al.*, 1968), and the 73% stimulation of incorporation of tracer [^{13}C]valine by a large dose of leucine (Smith *et al.*, 1994). Nevertheless, it has been useful with humans because improvements in mass spectrometry have permitted studies with ^{15}N or ^{13}C as tracer atoms.

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A complication of either method is that the true intracellular specific activity is not directly determinable in man; typically it is about 50% of that in plasma, because about half of the cytosolic amino acid pool, at least for lysine, tyrosine, and proline, is derived from breakdown of proteins in the liver. Several ingenious direct means have been devised to determine the specific activity of the intracellular free amino acid pool, presumably that pool from which amino acids are derived for albumin synthesis (Fig. 5-2). Some rely on the close involvement of intrahepatic arginine with the urea cycle, and link the label in arginine found in albumin to that in precursors of the guanidino portion of arginine such as CO_2 or α-NH_2 groups of amino acids. Originally McFarlane (1963) used the inexpensive label, $^{14}CO_2$, whereas Reeve *et al.* (1963) used [^{14}C]guanidinoarginine itself. In order to correct for the kinetics of the urea pool activities, the urea label turnover must be measured by injection of urea labeled with a different isotope or, alternatively, with the same isotope but at a considerably later time. The arginine–urea method is obviously applicable only to proteins made by the liver.

Heavy isotopes have now replaced radioactive ones in human applications. Olufemi *et al.* (1991) used [^{15}N]glycine or [^{13}C]leucine in the continuous-infusion method. Gersovitz *et al.* (1980), in a paper that is recommended for its clear description of the procedure, administered [^{15}N]glycine orally over a 60-h period then isolated albumin from the plasma by TCA–ethanol extraction. The ^{15}N in urinary urea reached a plateau after 36 h; ^{15}N in the albumin arginine guanidino groups, isolated by urease treatment after acid hydrolysis, climbed linearly from 0 to 60 h. This method has the advantage of avoiding a continuous intravenous injection of tracer, but has the drawback of requiring an oral dose every 3 h for the 60-h period!

Olufemi *et al.* (1990) have used compounds other than urea as a surrogate measure of the cytosolic amino acid specific activity. With [^{15}N]glycine, urinary hippurate glycine, and with [^{13}C]leucine, its deamination product, α-ketoisocaproate, have given reasonable results for albumin synthesis rates.

c. Circulating Proalbumin Levels to Indicate Albumin Synthesis Rate. The intracellular precursor of insulin, proinsulin, is found in the circulation in greater amounts when insulin secretion is stimulated (Robbins *et al.*, 1984); it is as though a slight spillover occurs with increased secretion. Methods sensitive enough to detect proalbumin in the normal circulation have not yet been developed. It might be worthwhile, considering the situation with proinsulin, to establish a sensitive assay; the necessary proalbumin-specific antisera are apparently available (Oda *et al.*, 1990). Such a simple index of the rate of albumin formation has been long sought after by those evaluating nutritional status, the condition of the liver after surgery, and the response to supportive therapy.

2. Absolute Rates of Albumin Synthesis in Vivo

In Table 5-3 are listed albumin synthesis rates as measured *in vivo* for rats and humans. Data for the rat were obtained with the pulse-label method; those

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TABLE 5-3
Rates of Albumin Synthesis Observed under Different Conditions in Humans and Rats *in Vivo*

Condition	Rat[a]				Humans		Reference
	Plasma albumin concentration (g/L)	Body weight (g)	Liver weight (g)	Albumin synthesis rate (mg/kg BW/day)	Plasma albumin concentration (g/L)	Albumin synthesis rate (mg/kg BW/day)	
Normal							
Young	33	245	10.5	828 ± 80	45	194 ± 37[b]	
Old	30	497	15.7	257	42	149	Gersovitz *et al.* (1980)
Young							
Fast 24 h	31	193	6.6	445	—	—	
Protein deficient	27	157	5.6	261	—	—	
Protein deficient, refed 24 h	28	≈190	≈9.5	1415	—	—	
Parenteral nutrition							
Glucose alone	—	—	—	—	30	157	Skillman *et al.* (1976)
Glucose + amino acids	—	—	—	—	28	243	Skillman *et al.* (1976)
Glucose + amino acids + lipid	—	—	—	—	31	385	De Feo *et al.* (1992)
Nephrotic	10	177	7.8	813	29	213	Ballmer *et al.* (1992)
GI protein loss	—	—	—	—	?	520[c]	Schomerus and Mayer (1975)
Diabetic	—	—	—	—	?	146	De Feo *et al.* (1991)
Acute hepatitis	—	—	—	—	?	61[c]	Mayer and Schomerus (1975)

[a]Values for the rat are from Peters and Peters (1972) and Schreiber and Urban (1978) except that the normal, young value is from a tabulation of five studies by Schreiber and Urban (1978).

[b]Average of five studies using CO_2–urea method (Jeejeebhoy *et al.*, 1969; Hafkenscheid *et al.*, 1973; Mayer and Schomerus, 1975; Gersovitz *et al.*, 1980; De Feo *et al.*, 1992) plus two using flooding method with [^{13}C]leucine (Ballmer *et al.*, 1990, 1992). The value of De Feo *et al.* (1992) was adjusted for relative lean body mass of 85%.

[c]Adjusted to bring reported value for normal humans (239) to average value of seven studies (194) for comparison.

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for humans were obtained either with the CO_2-urea procedure or the flooding method with [^{13}C]leucine described above.

To compare the normal synthesis rates of rats and humans, it must be realized that the rat liver is relatively larger than that of humans, 4.3 versus 2% of body weight (BW). Furthermore, the relative liver weight of the rat changes with the nutritional state and the liver weights for the human subjects were not known. Hence the data for the rat were converted from the mg/g liver/h of Table 5-2 to mg/kg body weight/day to be more comparable to the human data.

For the normal young man, multiplying the value of 194 mg/kg BW/day by 70/1000 gives 13.6 g/day of albumin synthesized. This agrees well with the average value of 13.3 g/day of albumin replaced in the steady state (see also Section III on Degradation). This is a replacement of 3.7% of the pool of 360 g in the whole body, or a half-life of 19 days.

The synthesis rate *per gram of liver* calculated from Table 5-3 is 193 (rat) versus 97 (human) mg/g liver/day, or 0.80 versus 0.40 mg/g liver/h, the more commonly used units in the laboratory. Hence the rat liver produces albumin at twice the rate of human liver on a weight basis, a not-unexpected difference considering the higher metabolic rate of the smaller animal. In the normal rabbit, albumin synthesis is about 350 mg/kg BW/day (Reeve *et al.*, 1963; McFarlane *et al.*, 1965), intermediate between that of rats and humans (Table 5-3); at 3.0% liver weight (Munro, 1969) this corresponds to 0.49 mg/g liver/h. Dixon *et al.* years ago (1953) documented the marked decrease in albumin turnover with increasing body size, the daily replacement rate falling from 58% in the mouse to 3% in the cow.

From Table 5-3 we also see that albumin synthesis falls off appreciably in the aged rat on a body weight basis. It drops 46% on a 24-h fast, and 68% on a protein-free diet. The highest value in the rat is found on the rebound from protein deficiency, 1415 mg/kg BW/day, almost exactly twice the normal rate and 5.5 times the rate just before refeeding protein.

In humans, synthesis falls less severely with age (Table 5-3), but is again markedly affected by nutrition. During parenteral nutrition without amino acids, albumin synthesis slows by 20%, rising to 385 mg/kg BW/day, double the normal rate, if amino acids plus an ample supply of calories in the form of both carbohydrate and fat are provided.

The highest synthesis rate reported in humans occurs with severe albumin loss through the gastrointestinal (GI) tract—520 mg/kg BW/day, equivalent to 36.5 g of albumin produced per day, 2.7 times the normal rate. Thus in rats and humans the liver can only increase its production by a factor of 2–2.7 on maximum stimulus, a not-surprising figure when it is considered that much of the protein-synthesizing machinery of the liver, about half of its total protein secretion, is already devoted to albumin formation in the normal state.

With major loss of albumin through the kidneys, the *in vivo* synthesis rate rises less than 10%. As will be noted in Chapter 6 (Section II.B,2), circulating

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toxins may hinder the liver in reaching its maximum output. The lowest recorded albumin synthesis rate in humans is 61 mg/kg BW/day, or just over 4 g/day, in a case of acute hepatitis.

II. DISTRIBUTION, FUNCTIONS, AND FATE IN THE BODY

A. Distribution

What happens to the albumin that is formed by the liver and discharged into the bloodstream? To follow where it goes, what it does, and its destiny, let us observe the wanderings of tagged albumin molecules freshly injected into the venous circulation.

Historically, iodinated albumin was first employed to determine plasma volume by Gibson *et al.* in 1946, whereas the first turnover and distribution study was by Sterling in 1951. A comprehensive review of albumin kinetics is in the masterly volume by Schultze and Heremans (1966). [For a detailed study of equilibration compartments in the rabbit, see Bent-Hansen (1991).]

When a tracer-size dose of homologous albumin is given intravenously, a characteristic pattern of label remaining in the plasma with time is seen, as in Fig. 5-8. The 100% point on the ordinate represents the concentration of the tracer in plasma after the labeled albumin has mixed completely with the intravascular volume but none has been lost from it. This point is obtained by sampling plasma every 5 min or so for three or four times, after a 12-min period for mixing, then extrapolating the relatively straight line back to zero time to compensate for the small loss from the circulation up to this time. This constitutes a determination of the plasma volume as well, because dividing the total albumin label injected by the fraction of label per milliliter of plasma gives the total milliliters of plasma, typically 40 mL/kg body weight.

The concentration of tracer falls rapidly during the first few days, then slows to an exponential decay. If these data are plotted on a semilog plot as in Fig. 5-8, extrapolating this line to zero time indicates the fraction of the label within the circulation, averaging 40% in many studies; the missing 60% is found to have moved to extravascular spaces. Thus, the amount of exchangeable albumin that is extravascular (177 g) is 1.5× that in the bloodstream (118 g), and the total exchangeable albumin pool (295 g) is 2.5× that in the bloodstream.

Figure 5-9 diagrams the major albumin pools and their exchanges. When albumin in extravascular fluids of tissues is determined directly, by extracting the tissue and performing immunochemical assays, larger amounts are found than the quantity calculated to be exchangeable (Table 5-4). The total extravascular pool measured directly is about 242 g, 2.05× the intravascular pool, and the total body albumin is 360 g. These figures are the ones used in Fig. 5-9.

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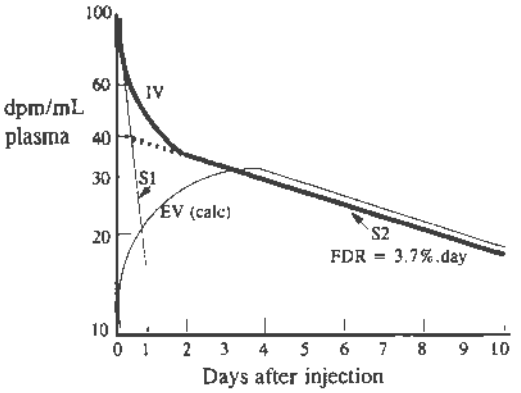


Fig. 5-8. Typical pattern of labeled albumin remaining in the plasma as a function of time after intravenous injection of a tracer dose of ^{125}I-labeled HSA. Slope 1 (S1) is the transcapillary escape rate (≈4.5%/h). The calculated curve for extravascular (EV) albumin is based on the work of Beeken *et al.* (1962). Slope 2 (S2) is the fractional degradation rate (FDR) of catabolism. The higher activity of the EV albumin during days 3–8 suggests that degradation occurs directly from the vascular compartment.

The slope of the slow exponential 1, Fig. 5-8, is about 3.7%/day in humans, $t_{1/2}$ = 19 days; this is the rate of metabolic degradation of albumin molecules, discussed later in Section III, and closely matches its synthesis in a steady state, 3.8%/day.

The rate of transfer to the extravascular space, known as the transcapillary escape rate (TER), is given by the steeper slope, exponential 1 in Fig. 5-8. Overall, a rate of 4–5%/h is obtained, for a half-time of exchange of about 15 h; the *average* molecule would leave the circulation every 22–24 h. This rate, however, like the representation of Fig. 5-9, is an oversimplification of the complex curve for the first few days. Many mathematical models have been applied

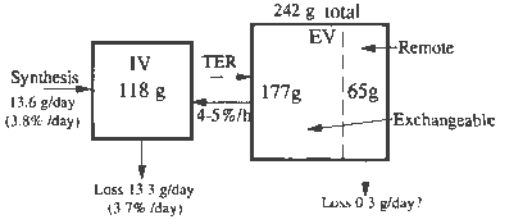


Fig. 5-9. Simplistic diagram of distribution of albumin in the body of a 70-kg human. The remote section of the EV compartment indicates the albumin detectable by chemical measurement but not available for exchange with albumin in the plasma. For a breakdown of the EV compartment see Table 5-4.

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TABLE 5-4
Distribution of Albumin in Body

Organ	Organ weight (fractional BW, rat)[a]	Extravascular albumin: Concentration in organ (g/kg)[b]	Extravascular albumin: Amount (g/70 kg BW)	Extravascular albumin: Fraction of total EV albumin	Extravascular albumin: Concentration in fluid (g/L)[b]
Skin	18.0%	8	100	41%	10–15
Muscle	45.5%	3	96	40%	10–15
Liver	4.1%	2	6	3%	–
Gut	2.8%	9	18	7%	25–30
Subcutaneous, *etc*	8%	4	22	9%	16
Total EV albumin			242[c]	100%	
			Intravascular albumin		
Plasma	4.0%		118	49%	42
Total EV and IV albumin			360		

[a]From Caster et al. (1956) and Katz et al. (1970a).
[b]Estimated from Humphrey *et al.* (1957), Katz *et al.* (1970b), and Worm *et al.* (1981).
[c]Of this 242 g, 177 g is considered to be exchangeable with circulating albumin.

to analysis of this equilibration (Reeve and Roberts, 1959; Beeken *et al.*, 1962), with three or more exponentials developed for as many extravascular subcompartments. A typical analysis yields a 12%/h equilibration rate for about 25% of the extravascular (EV) space and only 2.3%/h for the remaining 75%. The faster equilibration represents viscera, the slower, muscle and skin; experimentally a turnover of 2.1%/h was observed for the clearance of ^{131}I-labeled HSA injected subcutaneously in the human leg (Hollander *et al.*, 1961).

At a degradation rate of 3.7%/day, the life of the average albumin molecule would be 27 days (1/0.037). Entering the EV space every 23 h would mean that it makes 28 trips in and out of the lymph system during its lifetime. With 118/295 of the exchangeable albumin in the circulation at any one time, and a blood circulation time of 1 min, the average albumin molecule would also make about 15,000 trips around the circulation.

1. Locations of Extravascular Albumin

a. Extracellular Locations. Table 5-4 lists the occurrence of extravascular albumin in various organs, determined mainly by immunochemical analysis

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of human and rat cadavers but partly by equilibration of labeled albumin. The surprising finding is the large amount of albumin in skin—nearly half of the extravascular albumin, even though skin makes up only 18% of the body. An equal amount is in EV fluid of muscle, but there is much more muscle than skin. The average concentrations of albumin in the lymph from muscle and skin are about one-third of that of plasma, and in the lymph from the gut about two-thirds of the plasma concentration.

The extravascular spaces are not entirely permeable to albumin. In human skin albumin is excluded from 65% of the fluid volume (Bert *et al.*, 1986); in the skin of the rat this figure is 40–50% (Reed *et al.*, 1989). Permeability is greater into the subcutis than the dermis, but albumin does pass through the basement membrane and can be detected in the epidermis by immunofluorescence (Rabilloud *et al.*, 1988). Tendon is less accessible than skin, with 54% excluded volume in the rat, whereas muscle excludes only 26% (Wiig *et al.*, 1992).

The exclusion is caused by the network of collagen, proteoglycans, and hyaluronans, which constitutes the structure of extracellular spaces and which is relatively impermeable to macromolecules. Because albumin is excluded from about half of the fluid, its concentration in the accessible volume is elevated by the gel filtration effect, 33–35 g/L compared to 10–15 g/L in unrestricted lymph (Bert *et al.*, 1986). The content of albumin in skin rises with overhydration and falls with underhydration by 20–30% (Mullins *et al.*, 1987); thus the skin acts in a way as an albumin storehouse.

Albumin is found to some degree in every fluid of the body (Schultze and Heremans, 1966). In cerebrospinal fluid its concentration is low, 0.2 g/L, but amounts to 80% of the plasma protein present rather than only 60% as in plasma. The vitreous humour of the eye contains 0.7–2.4 g/L, but the aqueous humour contains only about 0.1 g/L. Amniotic fluid contains about 2 g/L (Bala *et al.*, 1987).

Albumin is also the major protein of transudates—pleural, peritoneal, and pericardial fluids—with a concentration of 10–15 g/L. In synovial fluid its concentration is about half that of plasma. The protein GCDFP-70 of breast cysts has been identified as albumin; in Type-I cysts its concentration is low, 0.3 g/L, whereas in Type-II cysts it is 10.2 g/L. In cystic fluids of ovarian tumors it is the major protein of endometrial and serous cystomas but is absent from some parovarian cysts (Mettler and Mader, 1992). In spermatoceles the albumin level is 1–4 g/L. Chicken serum albumin is found in egg yolks, where it is known as α-livetin (Szepfalusi *et al.*, 1994).

Secretions invariably contain albumin. Milk at the onset of lactation contains 5 g/L, falling to 0.7–1 g/L after a few days (Schultze and Heremans, 1966; Nagasawa *et al.*, 1973). Sweat, tears, and saliva contain 0.1–0.5 g/L. Albumin is the major protein in sweat, but only about 10% of the total protein of tears and saliva. Normal semen contains 0.8 g/L, the concentration increasing with increasing

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sperm count (Chard *et al.*, 1991); that the albumin in semen comes chiefly from the lower genitourinary tract is demonstrated by the higher concentration in prostatic massage fluids than in semen itself.

Urine normally has 0.025 g/L or less of albumin; much more than this is filtered through the glomerulus, about 1.5 g/day, but 99% of it is reabsorbed (and catabolized) by the proximal tubules. Before considering 1.5 g/day to be a sign of leakiness of the glomerular filter, it should be realized that this is only 0.004% of the 37,000 g of albumin passing through the kidney per day! [See also Chapter 6 (Section II,B,3,b).]

Albumin can be identified in the juices of the GI tract. Here it is a minor constituent, and is difficult to measure owing to the continuing process of digestion. Hepatic bile contains about 2 g/L of protein, of which about half is albumin (Schultze and Heremans, 1966); a small amount of this, ≈0.04 g, would have originated through release or leakage of newly formed albumin from hepatocytes (Section I,D,3). The albumin reaching the gut is degraded to amino acids that are reabsorbed, a process considered to be responsible for about 10% of the daily albumin loss.

b. Intracellular Locations. Within cells albumin is found in low concentrations, if at all. Hepatocyte cytosol contains none. About 0.5 g is confined in the secretory channels of the hepatocytes, and another 0.2 g within lysosomes in the process of degradation. Lysosome-confined albumin has been observed in aortic cells (Londoño *et al.*, 1992) and is probably fairly ubiquitous in actively metabolizing tissues.

Uncertainty still clouds the status of an albumin-like protein detected in muscle. It was identified as the β-actinin of rabbit muscle, a structural component of myofibrils (Muller and Heizmann, 1982), but lies mainly in the transverse tubular membranes (Knudson and Campbell, 1989). Evidence cited earlier (Section I,B,1) suggests that it is synthesized in muscle; a likely conclusion is that the "albumin" of muscle resides chiefly in the interstitium and may be involved in the delivery of fatty acids to support muscle metabolism.

Albumin observed in ovarian cells lies in lipid droplets of thecal, granulosal, and luteal cells and has been suggested to be engaged in delivery of steroid hormones or their precursors (Krishna and Spanel Borowski, 1989).

A 69-kDa protein identified in the seminiferous tubular compartment of the testis has been termed testibumin (Cheng and Bardin, 1986). It appears not to be albumin but to share some of its antigenic determinants. Another homologous protein, this one of 60 kDa, has been reported in the epididymus of rams (Carles *et al.*, 1992). The question arises whether these might be unrecognized members of the albumin superfamily.

Several sources have reported the detection of albumin within cells of the brain or peripheral nerves, particularly in nervous tissue still under development (Piñeiro *et al.*, 1982; Uriel *et al.*, 1982). It appears to have entered from the

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plasma rather than to have been synthesized *in situ* (Schachter and Toran-Allerand, 1982).

Albumin has been identified on lymphocytes and macrophages as the 70-kDa cell-surface-bound protein that constitutes the receptor for bacterial lipopolysaccharide (Dziarski, 1994); it can be shown to originate from serum-containing culture medium, but presumably occurs *in vivo* as well. Albumin detected in blood platelets as "tropomyosin-binding protein" (Hitchcock-DeGregor *et al.*, 1985) and albumin releasable from leukocytes on stimulation (Borregaard *et al.*, 1992) likewise are the results of uptake from plasma.

A trace of albumin is found in the cytosol of thyroid glandular cells, and even becomes iodinated there (Pretell *et al.*, 1968). It probably arises from the blood.

Albumin is 19% of the soluble protein of breast cancer cells (Soreide *et al.*, 1991), the level correlating inversely with the estrogen receptor content. A low albumin level in the cells thus predicted a positive effect of adjuvant tamoxifen treatment, and was an independent prognostic factor for relapse-free survival.

An intriguing occurrence of albumin is as the major "enamelin" protein extractible from developing bovine tooth enamel with EDTA with or without guanidinium chloride (Strawich and Glimcher, 1990). Identified by its 67-kDa size, immunoreactivity, and amino-terminal amino acid sequence, it constituted 70–80% of an enamelin extract.

2. *Mechanism of Escape from Circulation*

Albumin leaves the circulation through several mechanisms, which vary with the tissue involved. In organs having sinusoids—liver and bone marrow—plasma can pass through large gaps in the endothelium. Some other organs have fenestrated endothelia that allow unimpeded passage: the pancreas, small intestine, and adrenal gland are examples. Together these convection mechanisms account for about 50% of albumin transport from the circulation.

In the remainder of the body there is a continuous capillary endothelium. Starling's theory held that plasma proteins passed through this barrier as through a filter with a discrete permeability, at a rate that is the resultant of the hydrostatic blood pressure, the ambient pressure, the colloid osmotic pressure in the capillary, and the permeability of the wall. It now appears, however, that the approximately 50% of the albumin leaving the capillary lumen in these regions with continuous endothelium is transported by an active transcytotic mechanism. An albumin receptor has been isolated from the membranes of bovine lung endothelium, a 60-kDa glycoprotein first termed gp60 but now called *albondin* (Schnitzer and Oh, 1994). Serum albumin binds to this receptor and enters noncoated plasmalemmal vesicles of the endothelial cell; within 15 s it is discharged outside the cell on the side away from the capillary lumen, as demonstrated by the appearance of bovine albumin or gold-labeled mouse albumin in mouse capillaries (Milici *et al.*, 1987).

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The amount of the albondin receptor protein is reported to be 90 ng/cm^2 of membrane surface (Schnitzer *et al.*, 1988); the amount of bound albumin is 0.04 fmol/mg cell protein, which calculates to be 2.7 ng of albumin/mg cell protein (Smith and Borchardt, 1989). The apparent affinity for the receptor was given as 1 mg/mL (Schnitzer *et al.*, 1988), which would correspond to $K_A = 6.7 \times 10^4\ M^{-1}$.

Conformational changes in the lumenal albumin affect the binding and the rate of transfer. Addition of LCFAs in the physiological range of $\bar{\nu} = 1$–3 in the medium bathing monolayers of aortic or pulmonary arterial endothelium increased the rate of albumin transfer by 40% (Antohe *et al.*, 1993) to 200% (Galis *et al.*, 1988). Cationization [increasing the positive charge on the albumin by esterification with diamines or binding to protamines (Pardridge *et al.*, 1993)] caused as much as a sevenfold increase (Smith and Borchardt, 1989; Gandhi and Bell, 1992); the presence of arginine was particularly important (Powers *et al.*, 1989). Glycosylation of the albumin had a similar effect, perhaps again through an increased net positive charge.

The receptor-bound albumin itself alters the permeability properties of the endothelium to aqueous solutions. As it becomes part of the fibrous matrix, which is a sort of molecular filter, it decreases the permeability to other proteins (Huxley and Curry, 1985) and markedly reduces the hydraulic conductivity across cell monolayers (Dull *et al.*, 1991; Qiao *et al.*, 1993).

The distribution of the receptor protein, albondin, is widespread but selective. It is not present in capillaries of the brain (Pardridge *et al.*, 1985), in keeping with the low concentration of albumin in cerebrospinal fluid. Two other receptor proteins, gp18 and gp30, apparently act to bind albumin in preparation for its degradation and are discussed in Section III.

The placenta is a highly selective organ in its transport of plasma proteins. It has receptors that bind maternal immunoglobulin (Ig) G and protect it for delivery to the fetal circulation intact, whereas other plasma proteins are engulfed and degraded to free amino acids that are delivered to the fetus. Labeled HSA injected into a mother appears with no more than 5% of the maternal specific activity in the fetus after 25 days, whereas IgG reaches the full activity (Gitlin *et al.*, 1964). Other plasma proteins, including IgM, are degraded like albumin.

B. Functions

The importance of plasma proteins, particularly albumin, in stabilizing the physical environment of the blood has been recognized for over 70 years (Howe, 1925), but the significance of albumin as a vehicle for metabolites, as a protective agent, as a factor in lipid metabolism, and in miscellaneous, often bizarre, functions is still being recognized.

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1. Circulatory Roles

Only 60% of the mass of the plasma proteins, albumin is responsible for 80% of the colloid osmotic pressure of plasma (25–33 mm Hg). About two-thirds of this COP is the simple van't Hoff pressure, to which albumin contributes disproportionately because its molecular mass of 67 kDa is lower than that of the average of the plasma globulins, about 170 kDa (Scatchard *et al.*, 1944). The other third arises from the Donnan effect of the net negative charge of plasma proteins, which is essentially due entirely to albumin and its low isoelectric point (Figge *et al.*, 1991) (see also Chapter 2, Section II,B,2). Lundsgaard-Hansen has critically reviewed this role (1986).

Albumin accounts for essentially all of the macromolecular anion of plasma (Chapter 2, Section II,B,2) and supplies most of the acid/base buffering action of the plasma proteins. The slope of the albumin titration curve (Fig. 2-12d) in the physiological range is 0.13 mEq/g albumin/pH unit (Figge *et al.*, 1991), which means that the estimated 295 g of exchangeable albumin in the body would buffer 3.8 mmol of acid or base per 0.1 unit change in pH. In the bloodstream hemoglobin is, of course, a much more important buffer, but in extravascular fluids and, particularly, pools such as ascitic fluid albumin assumes greater importance.

2. Transport of Metabolites

a. Cargo and Routes. Ports of call and some of the cargo carried by albumin are seen in Fig. 5-10. Table 3-1 is a manifest of many of the endogenous compounds it transports; the nature of their loading was detailed in Chapter 3. The most important of these are the long-chain fatty acid anions, highly insoluble by themselves, and highly active metabolically with a turnover time of about 2 min (Spector and Fletcher, 1978). They are carried from the intestines to the liver, from the liver to muscle, and to and from adipose tissue.

Copper absorbed through the intestines is transported by albumin in the portal circulation, and is incorporated into ceruloplasmin in the liver (Gordon *et al.*, 1987). Some copper also binds to free histidine in plasma (Neumann and Sass-Kortsak, 1967); the $Ni(His)_2$ complex is much stronger than the analogous $Cu(His)_2$ complex, which slows transfer of Ni(II) to albumin (Tabata and Sarkar, 1992), and allows nickel to go primarily to the kidneys rather than to the liver.

Bile acids, particularly the more hydrophobic ones, are reabsorbed in the large intestine and transported by albumin back to the liver as part of their enterohepatic cycle.

For many hormones and vitamins, probably excluding folic and ascorbic acids, albumin acts not so much as a transport agent but as a mother ship or reservoir. More specific transport proteins are the primary carriers of steroid and thyroid hormones and of vitamins D and B_{12}, but they are present in only minute

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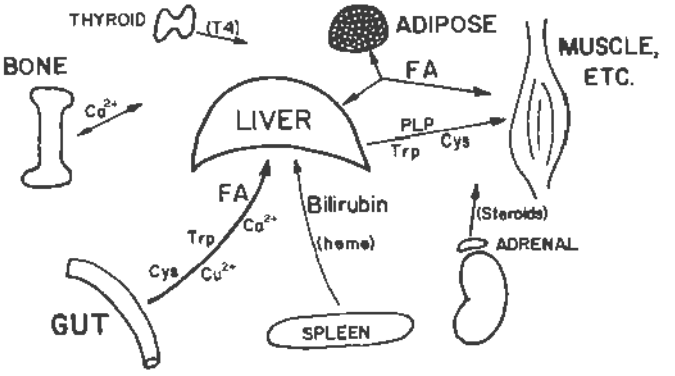


Fig. 5-10. Metabolic transport functions of albumin. Reproduced from Peters and Reed (1978) with permission of W. de Gruyter, publisher.

amounts whereas the larger supplies of ligand on albumin, although more weakly bound, serve to replenish them when their cargoes have been offloaded. The turnover times of these ligands on albumin are thus generally longer than those of the fatty acids.

A few compounds are transported as covalent ligands. Cysteine, homocysteine, and reduced glutathione would be rapidly oxidized by the dissolved oxygen of plasma, and are carried as mixed disulfides on Cys-34 of albumin (Chapter 2, Section II,B,5) and delivered to tissues (Awwad *et al.*, 1967; Lash and Jones, 1985). Pyridoxal phosphate transport as a Schiff base was described in Chapter 3 (Section I,E,2).

b. Delivery Mechanism. Is there a specific receptor mechanism for albumin by which it offloads its ligands? This question has intrigued investigators since it was raised by Weisiger *et al.* in 1981 in studies of the uptake of oleate by the perfused rat liver. The uptake increased when the $\bar{\nu}$ oleate/albumin was elevated, but reached a plateau when the albumin concentration was increased at constant $\bar{\nu}$; this was interpreted to reflect saturation of albumin receptors on the cell membrane.

Continued studies by Weisiger and co-workers (Pond *et al.*, 1992) and Schwab and Goresky (1991), dealing now with transfer of LCFAs to hepatocyte suspensions or to polyethylene sheets, support the concept that there is at least cellular "facilitation" of the uptake. The alternative, "conventional" model is release of the fatty acids from albumin by reversible dissociation followed by diffusion of the free fatty acid through an "unstirred layer" to the cell membrane (Smallwood *et al.*, 1988; Sorrentino *et al.*, 1989). Extraction of LCFAs by the liver is remarkably efficient, about 30–40% per pass, and whether the measured dissociation rates, $k_d \approx 0.045\ s^{-1}$ (Forker and Cai, 1992), are adequate to pro-

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vide enough free oleate, for example, to account for this uptake rate is not certain. Even though the time spent by an albumin molecule in a hepatic sinusoid is 8–10 s per pass, access to the cell membrane requires random movement into the space of Disse through holes in the sinusoidal lining; in other organs, such as adipose tissue and muscle, the typical time of passage through a capillary is only 1 s, posing a more stringent requirement.

The question has been approached with transfer of bile acids and dyes such as BSP and rose bengal, in perfused liver, hepatocytes, cardiac myocytes (Stremmel, 1988), adipocytes, and isolated cell membranes. Extraction efficiency with organic compounds rises with hydrophobicity (Tokumo *et al.*, 1991). ^{125}I-Labeled albumin has been found to bind, more or less specifically, to isolated cells, including erythrocytes (Ockner *et al.*, 1983) and activated T-lymphocytes (Uriel *et al.*, 1994); AFP bound to the lymphocytes as well. Hepatocytes (Reed and Burrington, 1989) and adipocytes had $(1–2) \times 10^6$ and 10^7 sites/cell, respectively. What is lacking is isolation of a receptor. Another complicating factor is the saturable, nonspecific binding of albumin, particularly if bearing a LCFA ligand, to surfaces of all kinds (Chapter 2, Section II,C,2,d), including glass fiber filters and the walls of laboratory containers. This binding can mimic receptors even when they are not present (Reed, 1990).

Binding to a surface, even a cell membrane, causes a conformational change in the bound albumin molecule (Horie *et al.*, 1988). Perhaps this surface-induced change is the basis of the receptor effect, the altered configuration having reduced affinity for a fatty acid and releasing it in the proximity of the membrane (Reed and Burrington, 1989). Carter and Ho (1994) likened the change to an **N** → **F** transition induced by a lower pH on the cell surfaces; a more likely transition would be to the **B** form, which can occur near pH 7.4 in the presence of calcium (Chapter 2, Section II,C,1,c). The ligand-free albumin, with reduced affinity for the surface, would then quickly be released. In this version of the uptake mechanism there is a degree of cellular "facilitation"—the albumin carries the fatty acid across the unstirred layer to the cell surface, obviating the need for spontaneous dissociation and diffusion of the free ligand through this region.

Once at the cell membrane, fatty acids can be readily absorbed into the lipid-rich matrix (Cooper *et al.*, 1989; Kamp *et al.*, 1993). On the cytoplasmic side they are picked up by one of several fatty-acid-binding proteins, unrelated genetically to albumin, which are widespread in tissues of many types (Ockner, 1990). An analogy is the transfer of LCFAs across the placenta; here the rate of uptake by the fetus is highly dependent on the concentration of albumin in the fetal circulation (Stephenson *et al.*, 1993).

The transport mechanisms of a few other ligands are in accord with the above concept. Albumin-bound testosterone is an example (Manni *et al.*, 1985). Even the transport of tryptophan, a weakly bound ligand, into brain may involve an enhanced dissociation mechanism (Pardridge and Fierer, 1990).

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The more hydrophilic hormones, T3 and T4, have dissociation rate constants from albumin of ≈0.6 and 1.3 s^{-1}, respectively (Mendel *et al.*, 1990; Whittem and Ferguson, 1990), which would seem adequate to allow unaided release in the ≈1-s passage through a tissue capillary. But the distribution of thyroxine within tissues becomes more uniform in the presence of albumin (Mendel *et al.*, 1987), suggesting some degree of interaction.

3. Protective Functions

a. Sequestration. In addition to the transport of foodstuffs, albumin acts as a toxic waste handler. It gathers bilirubin from sites of hemoglobin breakdown such as the spleen and delivers it to the liver for conjugation and biliary excretion. Normally this role, although constant, is a minor one because the $\bar{\nu}$ is less than 0.03, corresponding to the upper level of normal serum bilirubin of 10 mg/L. In the neonate, following intravascular hemolysis, and with liver disease the loading may be much higher (Chapter 6, Section II,B,6,a).

Bilirubin uptake by the perfused liver is proportional to the concentration of free bilirubin in the perfusate (Barnhart and Clarenburg, 1973), and evidence for the involvement of an albumin receptor has not been found (Stollman *et al.*, 1983). Its off-rate, $k_d = 0.03\ s^{-1}$ (Reed, 1977), should provide sufficient free bilirubin for its observed ≈ 18-min half-life in the circulation (Peters and Reed, 1978).

Hematin is also bound and delivered to the liver when its primary vehicle, hemopexin, has become saturated. Ligand-binding properties for hematin and bilirubin were considered in Chapter 3 (Section I,B).

Several exogenous toxins are sequestered by albumin and rendered harmless in the body. Benzene becomes an *S*-phenyl adduct to CySH-34 (Chapter 3, Section I,E,1), and the widespread carcinogen, aflatoxin G_1, is held in part covalently to a lysyl ϵ-amino group (Sabbioni and Wild, 1991) en route to destruction in the liver (Ewaskiewicz *et al.*, 1991). The hepatic carcinogen, *N*-sulfooxy-2-acetylaminofluorene, is converted to a noncarcinogenic sulfuric acid ester (Smith *et al.*, 1989). Many therapeutic drugs are sequestered (Table 3-5), with the result of both controlling their free, active concentrations and providing a reservoir for longer action.

b. As Antioxidant. Bilirubin, bound to albumin in the primary site, acts as an antioxidant to protect α-tocopherol from damage mediated by peroxyl radicals (Neuzil and Stocker, 1994). Protection was also evident for human cardiac myocytes in culture (Wu *et al.*, 1991), and a serum bilirubin concentration >12 μ*M* (0.7 mg/dL) in a group of young male subjects correlated with a 50% drop in observed coronary artery disease (Schwertner *et al.*, 1994). The bilirubin becomes converted to biliverdin in the process; recall that the bilirubin bound in the primary site is held in a twisted configuration and is more susceptible to effects of both oxygen and light.

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In the perfused rat heart, albumin lowers hydrogen peroxide levels and lessens injury of reperfusion (Brown *et al.*, 1989). Albumin also protects bound linolenic acid, free or in low-density lipoproteins, from peroxidative damage (Kozlov *et al.*, 1991). The albumin apparently sequesters lipid peroxidation products, thereby protecting the more sensitive apolipoprotein B from similar attack (Deigner *et al.*, 1992). With the lipoproteins the action results in a decrease in the fraction with rapid blood clearance.

The thiol group of Cys-34 is another site of protection against peroxidative action (Pirisino *et al.*, 1988), and was noted earlier (Chapter 3, Section I,E,1) to be the locus of nitric oxide transport in blood.

Albumin may in some ways not be protective but may aggravate formations of peroxyl radicals. Nickel(II) held by peptides with amino-terminal X-X-L-His, analogous to the copper–nickel binding site in albumin (Chapter 3, Section II,A), can trigger production of free oxygen radicals from peroxide, which then damage proteins in general and the histidine of the peptide sequence in the binding site in particular (Torreilles and Guërin, 1990).

4. Metabolic Effects

By virtue of its affinity for LCFAs, albumin acts as a recipient of fatty acids freed from lipids by enzymatic action. Hence it acts to stimulate lipoprotein lipase activity in adipose tissue (Campbell *et al.*, 1964) and milk, but appears to inhibit pancreatic lipase (Posner and DeSanctis, 1987). With lipoprotein lipase the hydrolysis of triglycerides is driven to completion rather than yielding mono- and diglycerides (Scow and Olivecrona, 1977). Release of LCFAs from circulating lecithin–cholesterol acyltransferase is stimulated (Tove, 1962), as is the release of lysophosphatides from cultured liver cells (Robinson *et al.*, 1989). In the latter instance albumin is binding a monoacyl ester, not a free fatty acid.

The actions of albumin in eicosanoid metabolism were given in Chapter 3 (Section I,A,4). Again they are attributable to binding of these sensitive lipid molecules. Albumin enhances release of arachidonate from macrophages, stabilizes certain types, such as prostaglandin PGI_2 and thromboxane $TBxA_2$, and affects the metabolic conversion of others, particularly favoring lipoxygenase and the dehydration of β-hydroxy keto forms rather than cyclooxygenase action.

Certain drugs are inactivated by albumin. Disulfuram, the antialcoholism drug, and the penem class of drugs were mentioned in Chapter 3. The extensive esterase activity associated with Tyr-411 (Chapter 3, Section I,D,6) is probably of little metabolic consequence. A mild fatty acid esterase activity of albumin has also been reported (Tove, 1962).

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5. Miscellaneous Functions

In vitro investigations of blood clotting and leukocyte activity mechanisms have divulged a number of apparent activities of serum albumin; by and large these do not merit the term "functions" but may be incidental effects. The presence of albumin, perhaps by a physical action, decreases the fiber thickness and network permeability of fibrin clots (Nair and Dhall, 1991). It also inhibits the aggregation of blood platelets induced by β-lactam antibiotics (Sloand *et al.*, 1992). The activity was not found if the albumin was completely defatted (Imada *et al.*, 1981), suggesting that a ligand may be responsible.

Albumin suppresses the spreading of neutrophils and their release of hydrogen peroxide on activation *in vitro* (Nathan *et al.*, 1993); this action was attributed to binding the agent, sialophorin, CD43, and blocking its release. A regulatory role in neutrophil function was also suggested by inhibition of their synthesis of leukotriene B_4 (Colli *et al.*, 1989), in this case probably also attributable to a lipophilic binding effect. The activation of macrophages by DBP *in vitro* has been reported to be inhibited by albumin (Yamamoto *et al.*, 1993).

Albumin carrying nitric oxide as an *S*-nitroso adduct has properties of endothelium-derived relaxing factor. *S*-Nitrosocysteine, which could be derived in this manner from the albumin–Cys mixed disulfide, is more active, but the albumin adduct has longer lasting activity (Keaney *et al.*, 1993).

The functions of albumin as a source of nitrogen nutrition to peripheral tissues is considered in Section III.D.

C. Survival in Analbuminemia

The genetic basis of the rare condition of analbuminemia was discussed in Chapter 4 (Section IV.E), and the 28 known cases, representing 24 families bearing what are probably independent mutations, are listed in Table 4-9. The analbuminemia is not complete; at least a trace of albumin is present due to gene leakage, and the condition is arbitrarily defined as a plasma albumin level less than 1 g/L. Reviews have appeared from time to time (Ott, 1974; Connode *et al.*, 1975; Dammacco *et al.*, 1980; Russi and Weigand, 1983; Kallee and Ott, 1992).

Although a small sample, the 28 analbuminemia cases provide a unique experiment of nature to study the role of albumin in the body. The subjects do amazingly well. The average age at detection was 24 years, and detection was in at least 9 cases incidental to some unrelated complaint. Followup information is sketchy, but only six albuminemics are believed to have died (Table 4-9), at ages 32, 55, 59, 68, 69, and 70 years, hardly the attribute of a deadly disease.

The most common finding is edema, particularly of the lower extremities; in some subjects it cleared with age. Fatigue, even collapse, is the second most common complaint. One patient received albumin therapy biweekly for over 40

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years, and still felt weak and tired before her injections. Seizures and agitation occurred in at least 5 of the 24 subjects, an abnormal incidence for which no basis is evident.

The circulatory dynamics are unusual. Colloid osmotic pressure (COP) ranges between 10 and 16 mm Hg compared to a normal of 25–33. It would be lower were not the other plasma proteins compensatorially increased; the average transferrin and α_1-antitrypsin levels are 7.1 and 5.8 g/L, respectively, two to three times their normal levels. Average total globulins are 52 g/L, compared to a normal of about 28. This strikingly low A/G ratio is the basis for the elevated erythrocyte sedimentation rate, which prompted investigation by serum protein electrophoresis in the first case.

Offsetting the low COP is a subnormal arterial blood pressure, averaging 102/63 compared to normal of 120/80 mm Hg. Although this hypotension will help to keep fluid within the vascular bed, it must contribute to the fatigue and weakness. Renin and aldosterone levels are about four times normal in response.

The outstanding blood chemical changes are related to lipid metabolism. The serum usually shows a gross hyperlipemia. β-Lipoprotein and total serum cholesterol average 8.5 and 3.6 g/L, respectively, well above the upper limits of normal of 3.4 and 2.2. In one subject the cholesterol measured 6.1 g/L. Yet atherosclerosis and arterial disease appear to be no more common than in the general population.

An associated finding, reported in at least 5 subjects but possibly occurring in others as well, is a marked lipodystrophy. The thighs and lower extremities are severely obese although the upper body appears normal (Fig. 5-11). The adipose tissue is resistant to attempts to shrink it by caloric restriction; a subject who dieted strenuously succeeded only in shrinking her upper torso to the point of emaciation, and the fatty tissue of the lower body was unaffected.

The reasons for this lipodystrophy would seem twofold, both the result of albumin lack. When LCFAs are cleaved from circulating lipoproteins by lipoprotein lipase in capillary walls, the free fatty acids are more prone to enter adipose tissue in the absence of albumin as a recipient. The edema of the lower extremities also attributable to the lack of albumin slows the return circulation from the legs, making it more likely that this adipose tissue will form there.

The Nagase analbuminemic rat (NAR) offers the opportunity to study this condition in a more invasive manner. The situation in rats resembles closely the human one, with low colloid osmotic pressure and lipemia. Like humans, the rats lead reasonably normal lives, bear offspring (Shumiya and Nagase, 1986), manage the stress of surgery, and show normal wound healing. The analbuminemic rats are able to stand protein deprivation as well as do their normal controls (Joles *et al.*, 1989a).

In the NAR, and in several human cases studied, androgen synthesis is depressed and the testes are small, accompanied in man by gynecomastia.

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Fig. 5-11. Lipodystrophy in a subject with analbuminemia. Reproduced from Barter *et al.* (1961) by permission of the Association of American Physicians.

Circulating prolactin was low in female NARs, and is believed to be the basis for their lower rate of mammary tumor induction following carcinogen administration (Nagase and Takahashi, 1987). The rats compensate for the lack of albumin better than do humans; their plasma globulins rise to provide a normal COP by adulthood, and the blood pressures are normal (Joles *et al.*, 1989b). Transplantation of liver cells from normal rats resulted in a normal level of circulating albumin (39 g/L) after 12 months, but, oddly, did not reduce the production of plasma globulins, and the total plasma protein concentration rose to 104 g/L (Ohta *et al.*, 1993).

Bilirubin transport capacity is reduced but is still 25% of normal, high-density lipoprotein assuming a role as its vehicle (Suzuki *et al.*, 1988). Still, penetration of bilirubin into the brain of newborn NARs is 1.6 times normal (Takahashi *et al.*, 1984). If the NAR is cross-bred with the Gunn rat to produce a strain lacking both circulating albumin and the ability to conjugate bilirubin, the offspring die with kernicterus within 3 weeks after birth (Takahashi *et al.*, 1984).

Drug transport is predictably diminished. In the NAR, salicylate has a higher elimination rate and broader tissue distribution characteristic of elevated levels of free drug (Hirate *et al.*, 1989). Both the NAR and human subjects show resistance to the diuretic, furosemide; albumin has been shown necessary for its delivery to the kidney (Inoue *et al.*, 1987).

The body attempts to compensate for its albumin lack by slowing the degradation of the small amounts that are present. In humans albumin half-life in

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plasma is increased from 19 days to 38–115 days (Cormode *et al.*, 1975), and in rats it rises from 3.5 to 8 days (Esumi *et al.*, 1979). If the circulating level is raised by albumin administration, the degradation rate rises to normal. The transit time of the small amount of albumin synthesized in the liver is normal in the NAR; an incidental finding is that the transit time for transferrin is shortened, perhaps simply the result of less competition by the normally greater amount of albumin in the secretory channels (Morgan and Peters, 1985).

Although the absence of albumin is compatible with life under the controlled conditions of civilization, there is evidence that the ability to survive in more stressful situations is impaired. Nagase rats kept without food at 5° survive only an average of 18 h, less than half as long as their normal counterparts (40 h) (Kawaguchi *et al.*, 1986). In seeking a function of albumin that might be responsible, these authors found that feeding of uric acid prolonged survival; the closest correlation would seem to be that the antioxidant action of uric acid substituted for that of albumin in ameliorating the toxic effects of the stress reaction.

Paralleling the diminished drug binding, a defect in sequestering toxic compounds can also be fatal. Male NARs are highly susceptible to developing bladder cancers when treated with *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine; the abnormally high urinary level of the carcinogen could be restored to normal by administration of albumin (Takahashi *et al.*, 1988). Another carcinogen caused widespread intestinal tumors in the rats (Ochiai *et al.*, 1991).

The rarity of analbuminemia may partly reflect an inability to survive the stress of fetal life. As Watkins *et al.* (1994b) noted, records of several analbuminemic families indicated loss of a sibling as a neonate. Nonimmunologic hydrops, a 98%-fatal fetal abnormality with an incidence of 1 in 3748 births, is marked by generalized edema with effusions and often polyhydramnios (Hutchison *et al.*, 1982; Iliff *et al.*, 1983). Hypoproteinemia was found in most of the fetal cases studied, and an albumin level less than 30 g/L in several. The implication is that many if not most cases of analbuminemia do not survive gestation.

The study of analbuminemia shows us that albumin as a major constituent of plasma is helpful in coping with stress and in containing environmental and physiological toxins. But why is ≈1 g/L of albumin invariably present? It appears to be enough to permit survival under favorable conditions, whereas the complete absence of albumin would be lethal. The small amount of albumin may perform critical functions of which we are not aware, perhaps as a messenger in receptor physiology, or as a regulator of genetic function.

D. Changes to Albumin in Circulation

Like any tramp steamer on its rounds, albumin molecules gradually accumulate effects of their exposure to the salty seas of the circulation during their

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average 27-day voyage. The effects are varied in nature and in general affect only a small percentage of the albumin molecules; they include "barnacles," "rusting," nicks and dents, and structural deformation.

Barnacles are illustrated by the numerous small compounds that bind covalently to circulating albumin. Most have been mentioned previously—cysteine and glutathione carried as mixed disulfides, vitamin B_6, δ-bilirubin, and glucose carried on ε-amino groups. Glucose normally is found with only 1% of albumin molecules, but a third or more carry mixed disulfides.

Penem compounds and the acetyl group of aspirin are among numerous drugs that modify circulating albumin. Ethanol is converted by the liver to acetaldehyde, which has been shown to bind to albumin; this depresses bilirubin binding (Karp *et al.*, 1985) and creates purported cytotoxins (Wickramasinghe and Hasan, 1991) under experimental conditions. Ethacrynic acid, a vinyl derivative, links by alkylation to the albumin thiol. Gold thiomalate and mercaptopurine bind to the thiol group as mixed disulfides. Albumin adducts of polycyclic aromatic hydrocarbons have been detected in sera of roofers and foundry workers and their measurement proposed as a marker of human exposure (Lee *et al.*, 1991).

Rusting occurs as oxidation. Traces of cysteic acid and methionine sulfoxide or sulfone are generally detectable in circulating albumin, and the proportion of SH-containing (mercapt)albumin declines with age (Leto *et al.*, 1970). *In vitro* studies on proteins demonstrate that metal-catalyzed oxidation (MCO) by free radicals, generated by cells from peroxide plus traces of Fe or Cu, degrades several species of amino acid side chains, including those of cysteine, arginine, lysine, tryptophan, and histidine (Meucci *et al.*, 1991; Stadtman, 1993). The damage is usually measured as poorly defined "protein carbonyls." Some tyrosine is converted to bityrosine. Albumin exposed to such cell-generated oxygen radicals has increased susceptibility to proteolytic hydrolysis (Davies *et al.*, 1987). These changes have not been demonstrated *in vivo*, and the strenuous conditions applied *in vitro* seem unlikely to occur even in inflammation except perhaps at a highly localized site. But oxidation of methionine to methionine sulfoxide has been measured in circulating α_1-antitrypsin of cigarette smokers (Brot and Weissbach, 1983).

Nicks in the circulating albumin molecule are seen as deamidation of asparagine and glutamine, proposed to be a cause of microheterogeneity (Spencer and King, 1971), and as loss of the N-terminal Asp or Asp-Ala of HSA, proved by sequencing (Brennan *et al.*, 1988) and resulting in a slower band on electrophoresis. Cleavage of internal peptide bonds is occasionally seen in pancreatic disease (Chapter 6, Section II,B,4).

Sogami *et al.* (1969) proposed that structural deformity caused by S–S interchange is one basis of microheterogeneity. A few years later Wallevik (1979) detected a change of isoionic point of 10% of ^{125}I-labeled BSA molecules injected into calves. The isomerization was compatible with that of S–S-inter-

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changed BSA and was reversible, in that BSA that had been S–S-interchanged *in vitro* would revert to the apparently normal form *in vivo*.

III. DEGRADATION: ROLE IN NUTRITION

Albumin degradation *in vivo* is usually determined by observing the breakdown of tracer-labeled preparations injected intravenously. The rate is obtained either by measuring the exponential curve of remaining labeled albumin in plasma after equilibration with the extracellular space, as in Fig. 5-8, or by measuring the loss of label from the body directly by its urinary excretion or indirectly by whole body counting (Mouridsen *et al.*, 1969).

For measuring degradation, the requirements for a tracer label are much more restrictive than they are for short-term studies of distribution as in Section II,A. Structural damage during isolation or labeling must be avoided because it results in shorter half-lives, i.e., faster degradation rates. Labels that can be reutilized to form more albumin are unsatisfactory; biological tracers such as [^{35}S]methionine or [^{14}C]leucine give falsely slow degradation rates.

Albumin is now usually labeled with ^{125}I. Its slower decay rate and lower energy are decided advantages over ^{131}I, which was popular before ^{125}I became available. ^{131}I may still be used if two proteins are to be studied simultaneously. I_2 coupled to albumin under gentle conditions, e.g., generation by peroxidase or Iodo-Gen at pH 7–8 (Chapter 7, Section IV,D,4), and added at an average of less than 1 I/albumin to minimize damage to the albumin apparently attaches chiefly at Tyr-411 (Peters *et al.*, 1988) and causes little damage to albumin structure. To prevent uptake of radioactive iodine by the thyroid gland, an excess of unlabeled iodide, 10 drops of saturated KI/day for humans, is often given to the subject during the experiment.

The longest half-lives obtained using radioiodine are taken to be the most valid ones. To date, the best result from a host of studies in man (Schultze and Heremans, 1966) appears to be the $t_{1/2}$ of 19.5 days using autologous HSA prepared under gentle fractionation conditions (Takeda and Reeve, 1963); a similar study using albumin prepared by cold-alcohol "Cohn" techniques yielded a $t_{1/2}$ of only 14.8 days, and earlier results ranged even below 10 days (Beeken *et al.*, 1962). I have chosen to use 19.0 for the $t_{1/2}$ of a young adult.

A. Rate of Degradation

The typical decay curve of labeled albumin in the plasma (Fig. 5-8) illustrates a study of albumin degradation as well as distribution. The fractional

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degradation rate (FDR), 3.7%/day, which corresponds to the $t_{1/2}$ of 19.0 days, and to catabolism of 13.3 g/days in a 70-kg man, or 194 mg/kg BW/day as shown in Table 5-3.

In the rabbit, the $t_{1/2}$ of iodinated albumin in plasma is 8 days (Reeve and Roberts, 1959); the turnover rates calculated from total excretion of iodine (urine and a small amount in feces) were in agreement, if time is allowed for breakdown and excretion to occur. In the young rat, albumin $t_{1/2}$ is 2–2.5 days (Reed and Peters, 1984). The higher values are obtained with labeled RSA that has been "screened" by injecting into a separate rat and using serum from this rat after 1–2 days, during which time rapidly degrading, denatured(?) forms are cleared (Katz *et al.*, 1961); the starting RSA must obviously have high specific radioactivity to be useful after such dilution.

The close fit of the slow component of Fig. 5-8 to a linear exponential line means that degradation is first-order or random. A fixed *fraction* of the plasma albumin is degraded daily, without regard for the age of the albumin molecules. Thus, the *amount* of albumin degraded daily is a function of the albumin concentration.

The daily degradation must be sensitive to factors other than the circulating albumin concentration as well, as elegantly demonstrated in Fig. 5-12. Andersen and Rossing (1967) first infused albumin into a volunteer for 30 consecutive days. This raised the plasma albumin level only from 40 to 55 g/L, but nearly doubled the total body albumin; the increase was mostly seen in the extravascular pool. The degradation rate increased more than twofold. In the next study, when the plasma concentration had returned to normal, plasma proteins were removed by daily plasmapheresis until the albumin level was 26 g/L. The result was the converse of albumin loading: the degradation rate fell markedly, along with a fall in the albumin "stores" as judged by the extravascular pool. The rate of albumin synthesis was affected only about half as strongly as was degradation in both parts of the experiment.

The fractional uptake of albumin by the perfused liver, like the loss of albumin from the circulation, is sensitive to the albumin concentration of the perfusate, falling markedly when hypoalbuminemic blood is perfused (Hoffenberg *et al.*, 1970).

In the studies of Fig. 5-12 the percent change in degradation is over three times the percent change in plasma albumin concentration. For a change of albumin level from 35 to 55 g/L, the predicted acceleration of degradation for a first-order reaction would by 60%. The degradation rate, however, changed from 6 to 20 g/day, or over 200%; it paralleled the total body albumin pool rather than the plasma concentration. Another factor affecting albumin degradation may be corticoids, which increase protein catabolism in general (Takeda, 1964; Sterling, 1960). Others are still unrecognized. Waldmann and Terry (1990) have studied a familial condition with hypercatabolism of albumin and IgG; perhaps this disease will give useful information.

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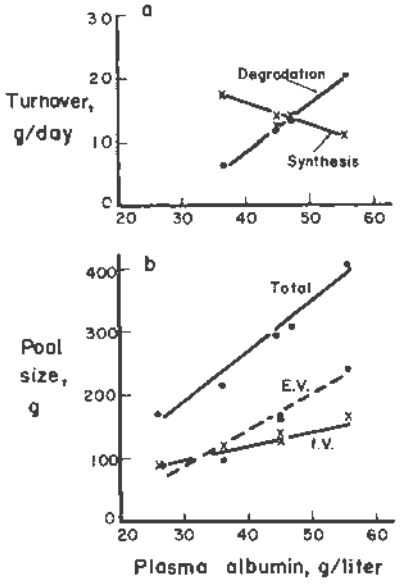


Fig. 5-12. Effect of plasma albumin concentration on (a) rates of albumin synthesis and degradation and on (b) distribution of albumin in the body. EV, Extravascular; IV, intravascular. Reproduced from Peters (1970) with permission of Academic Press.

B. Site(s) of Degradation

From the curves of Fig. 5-8, where the extravascular specific activity closely paralleled the intravascular, it appears that albumin breakdown occurs either directly from the vascular compartment or (Quincke and Maurer, 1957) from loci "with rapid access" to that compartment. This means simply that there is little if any pooling or delay between removal from the plasma and degradation.

In seeking the site(s) of this breakdown, the liver was first suspected owing to its high rate of protein metabolism. Screened RSA, however, was degraded by the perfused rat liver only to the extent of 15% or less of the total turnover (Katz *et al.*, 1961). Tracing injected albumin to different organs disclosed that the kidney degrades about 10% of the total but that most visceral organs such as spleen and lower intestine are minor contributors (Bent-Hansen, 1991). A maximum of about 10% is found to leak into the GI tract through the stomach and join the dietary protein (Jeffries *et al.*, 1962). Larger organs, muscle and skin, account for most of the disappearance. Some of the loss from the skin is as intact protein, recoverable in wash water (Brehm, 1966).

The ubiquity of albumin degradation in tissues was confirmed by ingenious use of "residualizing" labels; these are not degradable but remain trapped in

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lysosomes when their host protein has been digested. [^{14}C]Raffinose, a trisaccharide, and [^{14}C]sucrose tagged to RSA have shown muscle and skin to catabolize 40–60% of a dose of albumin (Baynes and Thorpe, 1981; Yedgar *et al.*, 1983). In muscle a study using dilactitol–^{125}I-labeled tyramine–RSA found fibroblasts to be the major cell type responsible (Strobel *et al.*, 1986). Uptake and degradation of ^{125}I-labeled HSA by activated T-lymphocytes has been noted (Torres *et al.*, 1992). Degradation of other plasma proteins—transferrin, fibrinogen (Hoffenberg *et al.*, 1970), and IgG (Henderson *et al.*, 1982) -appears to be as equally widespread as that of albumin, although at different rates.

C. Mechanism of Degradation

1. Pathway

The experiments with residualizing labels and later studies with the electron microscope also confirmed the presumption from earlier studies that plasma proteins are degraded by uptake into endocytotic vesicles that fuse with lysosomes to form secondary lysosomes. Albumin could be visualized entering the endosome–lysosome system of endothelial cells via bristle-coated pits (De Bruyn *et al.*, 1985).

Albumin is assimilated and degraded much more effectively if it is denatured or altered, particularly by compounds binding to ϵ-amino groups. The maleyl and formyl derivatives have been the most studied (Haberland *et al.*, 1989; Schnitzer and Bravo, 1993). They bind avidly to two albumin scavenger receptor glycoproteins in the cell membrane of endothelial linings, named gp18 and gp30 for their molecular size in kilodaltons. These scavenger receptors for modified albumins are widespread in tissues, including particularly liver (Ottnad *et al.*, 1992) and peritoneal macrophages (Zhang *et al.*, 1993).

Native albumin does not follow this pathway, but binds to the albondin receptor protein in plasmalemmal vesicles during its transcytosis through endothelium (Section II,A,2). It does not appear to enter the degradative endosome-lysosome system.

Within secondary lysosomes degradation proceeds most rapidly at pH 5–5.5; it requires a source of pyrophosphate bond energy (Beeken and Imredy, 1962). Pepstatin and *N*-ethylmaleimide inhibit albumin degradation in kidney cortical lysosomes, indicating that the enzymes chiefly responsible are aspartic and cysteine proteases, both active in the pH 5 region (Baricos *et al.*, 1987) and requiring thiol activation. Mego (1984) showed that thiol activation, particularly by reduced glutathione, was necessary to cleave albumin denatured by formaldehyde. If the disulfide bonds of the albumin were already reduced and blocked by alkylation, cathepsin D appeared to be the degradative enzyme. The ubiquitin degradative pathway (Hershko and Ciechanover, 1992) has not as yet been

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implicated in albumin degradation; it appears to be primarily concerned with proteins in the cytosol.

2. *Selection of Albumin for Degradation*

Modified albumin is degraded efficiently, whereas native albumin is taken into cells and released. What, then, is the signal for selecting an albumin molecule for degradation? Noted above (Section I,D) were various alterations to the albumin molecule in the circulation: oxidation, additions, and S–S interchanges. Polymeric forms have been found to be catabolized rapidly by Kupffer cells (Jansen *et al.*, 1991) and *in vivo* (Bocci, 1967). Derivatization with autoxidative products of arachidonic acid was effective in promoting uptake by macrophages.

Few of these alterations have been actually observed on circulating albumin. Yet they could still be the key to selection of albumin molecules for degradation. Because the kinetics of disappearance predict that degradation occurs promptly after removal of albumin from the circulation, modified forms could be removed so quickly, within a few 1-min circulation times, as to be undetectable. Because the modifications would occur randomly, without regard for the age of the recipient molecule, disappearance from the circulation would still follow the observed first-order curve.

Another influence on albumin degradation might be the protective effect of hydrophobic ligands on its configuration. LCFAs are probably the most important. Completely fat-free albumin molecules are more susceptible to proteolysis than those bearing one or two LCFAs. Even under normal conditions statistics show that a portion of albumin molecules are fat free (Table 3-3). When the LCFA/albumin ratio is elevated, as in analbuminemia, albumin degradation is greatly repressed. Other reversibly held ligands such as Trypan Blue have been found to depress albumin degradation by liver lysosomes (Davies *et al.*, 1971). An effect of rapidly exchangeable ligands would not be observable *in vivo*; however, coupling of palmitate covalently by affinity labeling increased the albumin half-life from 1.9 to 2.6 days in young rats at 3 M/M palmitate, but showed no apparent effect at 1 M/M (Reed and Peters, 1984).

D. Fate of Degradation Products

The end product of albumin degradation is its free amino acids. The possibility has been ruled out that fragments or peptides from partial degradation of albumin are used directly in albumin synthesis (Goldsworthy and Volwiler, 1958). Although a few fragments of albumin have been reported in blood and hemodialyzates (Kshirsagar *et al.*, 1984; Kausler and Spiteller, 1991), their significance is unclear.

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The final breakdown of small peptides may occur in the cytosol rather than in lysosomes because peptidases are concentrated there (Peters and Davidson, 1986). The released amino acids apparently mingle freely with the intracellular free amino acid pool, which, in turn, is in rapid (5–10 min) equilibrium with the plasma pool. They are not a preferential source of reincorporation into protein of the tissue in which they were produced (Radovich *et al.*, 1963), but join the body-wide pool of amino acids available for protein formation or catabolism. And so, like a rusty steamer cut up for scrap metal, degraded albumin contributes its substance to the body nitrogen supply; at 14 g/day it supplies about 5% of the protein turnover of the whole body.

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6

Clinical Aspects: Albumin in Medicine

Ample albumin in the plasma has been recognized as a sign of good health for almost a century. In this chapter I would like to consider the importance of albumin to medical practice—its measuremeot in body fluids, the significance of the albumin level, the relation of albumin to metabolic diseases, and the therapeutic and diagnostic uses of albumin preparations.

I. ASSAYS

Early assays for albumin were based on its physical properties, the albumin being physically separated from the globulins by its high solubility or its rapid electrophoretic migration. These assays have been almost entirely supplanted by methods utilizing the chromogenic effect of albumin on ligands or its reaction with antibodies. Because most of the methods give relative answers only, the calibrating standards must be chosen with care as well. For more detailed information on methods, reviews by Watson (1965), Walsh (1983), and Hill (1985) are recommended. A readable account of the history of albumin and other plasma proteins in clinical chemistry is that of Rosenfeld (1982).

A. Methods

Howe's (1921) 21.5% (1.5 *M*) sodium sulfate procedure was the prevailing albumin/globulin method for several decades, and was the one cited in

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Peters and Van Slyke's authoritative textbook, "Quantitative Clinical Chemistry," in 1932. Various concentrations of sodium sulfate, with or without sodium sulfite for better solubility of the salts, have been proposed; Watson (1965) recommended 1.8 *M* sodium sulfate as giving a cleaner separation. Ammonium sulfate at 2.45 *M* also gives a sharper separation of globulins (Herken and Remner, 1947), but the need for nitrogen analysis or the biuret reaction to measure the resulting protein fractions precluded the use of ammonium salts.

Despite such refinements the separation of albumin and globulins is incomplete and some globulins are included in the "albumin" fraction. Its purity is affected by the A/G ratio, as well as by the amount of shaking, the temperature and time allowed for precipitation, and the effectiveness of the necessary filtration or centrifugation. Kingsley (1940) introduced the use of a small amount of ether to accelerate flocculation of the globulins; such use clearly predated the era of governmental laboratory safety mandates.

The ability of albumin to withstand organic solvents under acid conditions has been useful for its separation from globulins. The most popular reagent was 1% TCA–96% ethanol (Debro *et al.*, 1957), later modified to hydrochloric acid–ethanol (Fernandez *et al.*, 1966); the technique had been introduced as early as 1932 by Race and probably provided the most accurate albumin values of the day. The albumin is too dilute to measure in the organic supernatant, but can be precipitated by sodium acetate, or the globulins can be assayed in the precipitate and the albumin value obtained by difference from the total plasma protein value.

Electrophoretic separation of albumin has been employed since Arne Tiselius published his doctoral work in 1937. In his system of "free" electrophoresis, the albumin boundary leads the plasma proteins in the anodic direction and its concentration can be measured by UV absorption or by refractive index change with the Schlieren technique; Dole (1944) published early clinical findings. E.L. Durrum (1950) introduced the use of solid supports for electrophoresis of serum proteins, with the run time extended until there is actual separation of zones of protein fractions from plasma that had been applied as a narrow band. Hence the name "zone" electrophoresis has been applied to this technique. It is useful for both analytical and preparative separations.

In zone electrophoresis the separated proteins are detected by staining with any of several wool dyes such as Amido Black or Ponceau Red, after fixing them to the support with TCA or sulfosalicylic acid. As a support Durrum used filter paper; the affinity of albumin for cellulose of paper, however, causes a faint blanket of albumin to be retained along its path, yielding low albumin but slightly high globulin values (Peeters, 1959). A thin film of cellulose acetate succeeded filter paper in the 1960s (Brackenridge, 1960) and is still widely used (Keyser and Watkins, 1972) (e.g., Fig. 4-5); thin agarose gels are also popular.

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The latest innovation is capillary electrophoresis, performed in narrow but long (e.g., 27-cm) tubing with detection by UV absorbance (Pande *et al.*, 1992). Predictably, care must be taken to overcome the tendency of albumin to adsorb on the capillary walls.

In electrophoretic assays albumin is defined simply as the major anodic peak. This is an arbitrary definition, because traces of free insulin and amylases actually migrate with albumin, and small amounts of α_1-globulins may be included in the zone selected for assay. The effect of these contaminants is minor, however, and electrophoresis has been often regarded as the standard of comparison for other albumin methods in the clinical laboratory.

All of the above procedures are labor intensive and time consuming, and methods not requiring physical separation have been sought. Among some creative approaches are differential pulse polarography (Alexander and Shah, 1980), hydrolytic action on fatty acyl aryl esters (Gürakar and Wolfbeis, 1988), and colorimetric assay for tryptophan, which is assumed to be largely a constituent of globulins (Saifer and Marven, 1966), the albumin then being calculated by subtracting the globulins from the total serum protein value. The preponderance of current methods, however, utilize either a spectral change effected on binding of an aryl ligand or a reaction with antibodies.

The use of chromogenic dyes rose from the "protein effect" caused by albumin on several colorimetric pH indicator compounds, the first apparently being methyl orange (Brncken and Klotz, 1953). It gave high results (Lundh, 1965), and other dyes followed. HABA (Rutstein *et al.*, 1954) quickly became popular, but in time was found to suffer from competitive binding by drugs and from interference with its yellow color by bilirubin and hemoglobin in serum (Ness *et al.*, 1965; Arvan and Ritz, 1969). BCG had been introduced by Rodkey (1964) as a reagent for albumin assay at pH 7.1; the high blank reading of the reagent discouraged application at this pH. Soon a BCG procedure using pH ≈4, where the blank is very low (Bartholomew and Delaney, 1964), became the dominant albumin method; a nonionic detergent, e.g., Brij-35, is needed to prevent precipitation of the BCG–albumin complex at this pH. This procedure as refined by Doumas *et al.* (1971) at pH 4.2 is rapid, flexible, and sensitive, $A_{628\ nm}$ at 1 g/L albumin = 28.4, allowing 1:200 dilutions of plasma.

But problems appeared with this BCG procedure as well. Some globulins, particularly lipoproteins, also react with BCG, although less rapidly than does albumin. This effect causes falsely high albumin values, particularly in serum from pathological cases in which the A/G ratio is low—the very region in which albumin data are the most important. Specificity for albumin is now largely achieved by measuring the color change after only 30 s or less, before there is time for appreciable reaction with globulins (Webster, 1977).

Prompt colorimetry is impractical with many automated machines, so another indicator dye, BCP, is widely used as well. Introduced as an albumin

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reagent by Louderback *et al.* (1958), BCP has been shown not to be as reactive with globulins as is BCG (Pinnell and Northam, 1978). None of the dyes is the perfect answer for albumin analysis; δ-bilirubin (Ihara *et al.*, 1991) competes with BCP, and heparin in high concentrations (Hallbach *et al.*, 1991) causes high values with BCG. Ligands showing fluorescence changes, such as ANS (Rice, 1966) and two cyclopentene derivatives (Kessler and Wolfbeis, 1992), have been proposed, but require specialized detectors for the fluorescent emission.

Immunochemical procedures overcome most of the shortcomings of the dye-binding methods and are rapidly replacing electrophoresis as the gauge against which other assays are compared. Their specificity is limited only by the quality of the antiserum employed. Techniques that observe precipitation between albumin and antialbumins in agar, such as radial immunodiffusion (Mancini *et al.*, 1965) or Laurell's "rockets" (1966), tend to be slow. Measuring the turbidity of immune precipitates in suspension (Schultze and Schwick, 1959) is faster, but is less sensitive than measuring the early stages of their formation using light scattering with nephelometry (Keyser *et al.*, 1981); the latter has become the method of choice in laboratories with the necessary detection equipment. The highest sensitivity is seen with blocking reactions, in which added albumin displaces an antibody-bound albumin derivative; the released derivative is then detected with amplification by fluorescence or enzyme-generated chromphores (Mueller *et al.*, 1991).

For measuring albumin in plasma and pleural or peritoneal fluids, where its concentration is 10 g/L or higher, the BCP or rapid BCG methods are in general use. Sample sizes can be as small as 5 μL with most automated equipment or with "kits" of reagents available from major biochemical supply houses. In nationwide quality-control programs, performance of these two colorimetric procedures has now reached a precision of ±0.5 g/L (SD) at ≈35 g/L, and an accuracy within the CLIA (Clinical Laboratory Improvement Act of 1988) Fixed Limit Goal of ±0.9 g/L at the same level.

Albumin is highly stable in plasma, and specimens can be stored for several weeks at 4°or several days at 23° before assay if evaporation is prevented; the protease inhibitors of plasma appear to provide protection against breakdown. For longer storage, freezing at −70° is recommended, with precautions against loss of water vapor and with thorough mixing on thawing.

BCG or BCP methods are sufficiently sensitive to measure albumin in cerebrospinal fluid, which resembles a filtrate of plasma. A lesser dilution, ≈1:5, is employed rather than the 1:200 used with plasma, because the spinal fluid albumin concentratioo is normally only ≈200 mg/L.

Urine is a much more complex mixture as compared to spinal fluid, containing many potential interfering substances. Dipsticks with immobilized BCG are useful as office or bedside tests for markedly abnormal urines, but

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such dye-based procedures lack the sensitivity to measure urinary albumin in its normal range of <25 mg/L, and so immunochemical procedures are required. Of these, several approaches are suitable for measurements with sensitivity as low as 1 mg/L. Imprecision is much higher than with plasma samples, however, ranging to nearly 50% with dilute specimens (Mueller *et al.*, 1991). As pointed out by Bakker (1988), the nonspecific binding of albumin to polystyrene or glass surfaces (addressed in Chapter 2, Section II,C,2,d) is a concern at these low levels; it can be obviated by adding a detergent such as Triton X-100 to the reagents.

Clinical chemists disagree over the stability of albumin at low concentrations in urine specimens. It appears clear that albumin in urine is stable for some hours at 23° and for a few weeks at 4° without preservative. Buffering the pH near 7 improves the stability (Townsend *et al.*, 1988). In saving specimens for longer periods, as in epidemiological surveys, the evidence is contradictory; losses seem to occur at −20°. Giampietro *et al.* (1993) recommend −70° for prolonged storage.

B. Standardization

To calibrate dye-binding properties and immunochemical methods in the clinical laboratory, a solution of pure human albumin with known concentration is required. Bovine albumin and albumins of other species are unsatisfactory, because both dye-binding properties and immune determinants vary among species.

Specifications have been publicized for a primary human albumin standard (Hobbs *et al.*, 1979), and promulgation of a human albumin standard solution by the United States National Institute for Standards and Technology analogous to its 70 g/L BSA preparation, Standard Reference Material (SRM) No. 927, is pending. Meanwhile, an HSA preparation meeting these specifications is available from Bayer (formerly Miles Laboratories, Inc.), and satisfactory HSA calibrator solutions are available from several laboratory supply firms.

If an HSA calibrator is to be prepared locally, HSA fraction V is recommended over crystalline preparations, which may contain crystallization aids that interfere with dye binding (Ness *et al.*, 1965). Crystalline preparations also tend to include more dimers and oligomers, which are a source of errors in immunoprecipitation procedures (Blaabjerg and Hyltoft, 1979). The HSA content of a solution of fraction V can be determined by a biuret procedure for total protein, with BSA as a standard (Chapter 7, Section III,D), and the small amount (<4%) of globulins can be determined by electrophoretic assay. For instruction on preparing albumin solutions see Chapter 7 (Section IV,A).

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II. PATHOLOGY: CHANGES IN DISEASE

A. Albumin Concentration and Its Significance

1. Normal Levels

The albumin concentration in the blood serum of an average adult human is 42.0 ± 3.5 g/L (SD), with a range of 35–50 g/L (Table 6-1). Earlier estimates using the less specific method of Howe gave 47 g/L. The above figures are from Rochester, Minnesota, and Bethesda, Maryland; mean levels found in a study from Boston and Copenhagen were 40 and 47 g/L, respectively (Greenblatt, 1979; Weeke and Krasilnikoff, 1971), the variations probably representing nutritional differences. In electrophoretic analyses the albumin normally comprises 60 ± 4% of the total serum proteins; their normal concentration is 70 g/L.

The conditions under which a blood specimen is drawn affect the observed concentration of serum proteins. Serum albumin levels do not differ in arteries

TABLE 6-1
Normal Albumin Concentrations in Serum with Age[a]

Age	Serum albumin level (range) (g/L)		
	Fetus		Mother
12 weeks	10.9		35.0
26 weeks	27.1		27.1
40 weeks	35.1 (36–44)		30.5
	Child		
0.3 year	37.8 (30–45)		—
To 1 year	42.2 (35–47)		—
To 5 years	46.2 (38–50)		—
To 10 years	45.2 (30–55)		–
To 13 years	42.5 (35–49)		—
	Male	Both genders	Female
Adult	43.0	42.0 ± 3.5	41.0
70 years	41.6	—	41.1
80 years	41.3	–	
90 years	38.5		38.9

[a]Data for fetus and child from Krasilnikoff and Weeke (1971) and Krauer *et al.* (1984); adult data from Keating *et al.* (1969), Campion *et al.* (1988), and Salive *et al.* (1992).

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and veins (in rabbits, anyway) (Kawaguchi, 1977), but leaving a tourniquet on a vein causes the albumin level to rise with time, more than 15% after 3 min. Subjects in a prone position show albumin levels 7% lower than those in the usual sitting position for venesection, and levels in standing subjects are 3% higher owing to hemoconcentration of the plasma proteins (Statland *et al.*, 1974). There is a slight diurnal rise, which is probably activity related, with the concentration rising 1–2 g/L during the day to a peak near 6 pm (Statland *et al.*, 1973). The value for a particular individual remains remarkably constant, the change from day to day, termed biological variation, averaging only 2.5–3%.

A weakly significant difference in serum albumin level is seen with gender. Females average about 2 g/L less than males in middle age but the difference disappears by age 70 (Table 6-1). A milder degree of hypoalbuminemia, <35 g/L, is seen in women on oral contraceptives (Weindling and Henry, 1974); recall the negative effect of estrogens on albumin synthesis. Administration of testosterone to nonathletic men for 6 months, in pharmacological doses sufficient to raise the circulating testosterone level by 90%, also resulted in a decrease (4–5%) in their serum albumin concentration (Young *et al.*, 1993).

The effects of age are slight after the first year (Table 6-1). After a peak concentration of 46 g/L at ages 1–5 years and a well-maintained plateau of about 42 g/L at 20–40 years, the serum concentration gradually falls at a rate of ≈0.05 g/L/year to ≈39 g/L at >90 years of age (Campion *et al.*, 1988). The rate of decline is slightly greater in men, 0.08 g/L/year, than in women, 0.04 g/L/year (Keating *et al.*, 1969), so that in the tenth decade the albumin concentration in women exceeds that in men.

The earliest measurements on fetuses were 11 g/L at 12–15 weeks (Table 6-1). After that time the fetal albumin level rises steadily at a rate of ≈0.9 g/L/week (Cartlidge and Rutter, 1986) to reach 35 g/L at parturition. Meanwhile the mother's albumin has fallen, from 35 g/L at 12 weeks to a low of 27 g/L at 26 weeks, recovering by 40 weeks to 30.5 g/L but still 15% lower than that of the fetus. The interplay shows the ability of the fetal liver to produce albumin when given sufficient free amino acids through the placenta, and the drain on the mother's system from increased placental degradation of her own plasma proteins to supply amino acids for the child. Another hypothesis relates the decline in maternal albumin level to negative feedback of maternal AFP on albumin production, because the maternal albumin and AFP concentrations show an inverse relationship (Maher *et al.*, 1993).

Preterm infants of 26- to 28-week gestation have albumin levels of 19–20 g/L (Cartlidge and Rutter, 1986; Reading *et al.*, 1990), lower than might be predicted from Table 6-1. For the first few weeks of neonatal life their albumin concentration rises at about the rate it would have sustained *in utero* and then gradually joins the range for full-term infants. The snbnormal level was not correlated with occurrence of edema.

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In the cerebrospinal fluid the level of ≈200 mg/L is constant through adulthood and falls only slightly with age, in accordance with the fall in the serum level (Garton *et al.*, 1991). In the amniotic fluid, albumin concentration ≈2 g/L, the ratio of surfactant to albumin is used as a screening test for fetal pulmonary maturity (Bayer-Zwirello *et al.*, 1993).

The quoted urinary albumin of <25 mg/L is the upper limit of normal. In early-morning urine a study of 5670 adults showed an average content of 6 mg/L, with an upper limit of normal (±2 SD) of 28 (male) and 29 (female). In relation to creatinine output rather than to urine volume the figures were 2.3 and 2.8 g albumin/mol creatinine (male and female, respectively) (Metcalf *et al.*, 1992). When measured as excretion rate, another study found <8 μg/min, corresponding to 11.5 mg/day, for 25 subjects (Bakker, 1988). Urinary albumin increased slightly with increasing diastolic blood pressure, body mass, serum triglyceride concentration, acute exercise (Kramer *et al.*, 1988), alcohol intake of more then 50 g/day, or cigarette smoking (Metcalf *et al.*, 1993); the latter effect was maximal at a half pack a day. The use of urinary albumin excretion data to detect incipient renal damage is discussed later, in Section II,B,3.

2. *Diagnostic and Prognostic Value*

Hyperalbuminemia, serum level >55 g/L, is seldom seen in the absence of dehydration. Even under optimal nutritional conditions albumin synthesis reaches a plateau (Chapter 5, Section I,E,2), and as the albumin concentration rises its degradation accelerates markedly (Fig. 5-12). A high-normal level is associated positively with body weight and body fat in men (but not women) (Micozzi *et al.*, 1989), and with birth weight, neonatal survival, and growth rate in piglets (Stone and Leymaster, 1985; Wise *et al.*, 1991). The albumin level was recently found to vary directly with arterial blood pressure, a 10-g/L rise corresponding to a 1.8-mm Hg rise in systolic and 0.8-mm Hg rise in diastolic pressure (Hu *et al.*, 1992).

Hypoalbuminemia is by far the more common condition. It can result from decreased synthesis (malnutrition, liver disease), increased degradation (nephrosis, GI losses), or increased loss from the circulation to the "third space" (shock, edema). Luetscher observed in 1947, "the common denominator of almost every pathological state is a relative or absolute decrease in the serum albumin." This volume cannot dwell on detailed findings, and reviews by Watson (1965), Rosenoer *et al.* (1977), and Doweiko and Nompleggi (1991a) and clinical textbooks are recommended for further reading.

The lowest circulating albumin values, <20 g/L, are characteristic of nephrosis and protein-losing gastroenteropathy, and have been noted with sepsis in the presence of anergy (Harvey *et al.*, 1981). Slightly higher levels, 20–23 g/L, occur with severe hepatic cirrhosis and glomerular nephritis. Values

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between 23 and 30 g/L are frequently seen in the acute-phase reaction, viral hepatitis, malnutrition, carcinomatosis, rheumatoid arthritis, and severe infections. Two studies noted low albumin levels in delirium (Trzepacz and Francis, 1990; Dickson, 1991); in this situation accompanying nutritional factors may have been responsible.

Persistently severe hypoalbuminemia, <25 g/L, is associated with observable changes in the fingernails. Muehrcke's lines are thin, paired, stationary white bands across the nails parallel to the lunule (Muehrcke, 1956), while the yellow-nail syndrome (Sahi and Bansal, 1988) is characterized by lymphedema and serous effusions.

The prognostic value of serum albumin levels is being increasingly appreciated. Large surveys show that hypoalbuminemia is as ominous a predictor of cardiovascular disease as is smoking (Phillips *et al.*, 1989). An albumin level of <44 g/L was associated with a doubling of the incidence of coronary heart disease in men and a 56% increase in women in the age range 45–64 years (Gillum and Makuc, 1992). Middle-age men with albumin >48 g/L died from all causes at an annual rate of only 4/1000, compared to 23/1000 for those with albumin <40 g/L (Phillips *et al.*, 1989). The causes included as-yet undiagnosed cancer; albumin levels showed a highly significant negative association with long-term cancer risk (Stevens, 1990). In 2300 older men and women, 50–89 years of age, hypoalbuminemia appeared to be a detector of subclinical disease; for every standard deviation decline in concentration (3.5 g/L) the relative odds of dying were increased by 24%, even when the findings were corrected for smoking, alcohol consumption, and exercise (Klonoff-Cohen *et al.*, 1992).

The albumin level is even more prophetic in nursing home residents. Death rates for men 40–96 years of age were 50, 43, and 11% per annum for groups with albumin levels <35, 35–40, and >40 g/L, respectively (Rudman *et al.*, 1987). In 4100 men and women >70 years old, a higher albumin correlated with a lower mortality rate, with the effect even extending into albumin levels above the usual normal of 42 g/L; an albumin concentration of >43 g/L predicted a 20% reduction of mortality in men and 40% in women relative to a concentration of 41–43 g/L (Corti *et al.*, 1994).

On hospitalization, in 15,500 patients older than 40 years, the 21% having albumin <34 g/L had longer hospital stays, more readmissions, and higher mortality (14% compared to 4%) than patients with normal levels (Herrmann *et al.*, 1992). After a hip fracture, the 11-month mortality was 70% for patients with albumin <30 g/L and only 18% for those with a higher albumin level (Foster *et al.*, 1990).

Many prognostic studies have focused on specific diseases. Albumin concentration is one of the most accurate indicators of progression in multiple myeloma (Bladè *et al.*, 1989; Chen and Magalhaes, 1990), where patients with stage-III myeloma had albumin <38 g/L, and a level below 30 g/L identified unequivocally

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advanced disease. Mortality and morbidity in chronic ambulatory peritoneal dialysis (Rocco *et al.*, 1993; Avram *et al.*, 1994), Hodgkin's disease (Gobbi *et al.*, 1985), primary biliary cirrhosis (Balasubramanian *et al.*, 1990), malignant melanoma (Sirott *et al.*, 1993), and following cardiac surgery (Rich *et al.*, 1989) were strongly predicted by albumin levels. Hypoalbuminemia is associated with increased odds of the need for an operation in Crohn's disease (Makowiec *et al.*, 1991) and of risk of developing necrotizing enterocolitis in infants (Atkinson *et al.*, 1989). In HIV-infected patients, low serum albumin was also the best predictor of operative mortality (Binderow *et al.*, 1993), and foreshadowed impending death independently from CD4 counts (Guenter *et al.*, 1993).

B. Relation of Albumin to Some Metabolic Diseases

1. Malnutrition

The close dependence of albumin synthesis on the amino acid supply to the liver has been stressed repeatedly in this volume. When the rate of synthesis falls due to malnutrition, the decreasing circulating albumin level causes movement of extravascular albumin to the bloodstream as well as slowing of albumin degradation (Fig. 5-12) in compensation. Thus, the observed change in serum albumin level only very slowly comes to reflect the protein intake.

The severest form of protein malnutrition occurs among residents of poorer countries who receive ample caloric intake from foods such as yams, but little or no protein. In children, who are the most seriously afflicted, the result is the disease termed kwashiorkor ("red boy" in Swahili). When the circulating albumin level falls below 20 g/L, edema and ascites become marked and the prognosis is bleak. An albumin concentration less than 16 g/L has been found by multivariate analysis to be the most accurate predictor of risk of death in this tragic condition, more so than weight, triceps skin-fold thickness, upper arm circumference, or edema (Dramaix *et al.*, 1993).

In total starvation the fall in albumin concentration is less marked than in protein depletion with an adequate calorie supply. The zero caloric intake depresses the insulin level, which causes breakdown of protein for energy, first in liver, then in muscle, so that amino acids are still available (Lunn and Austin, 1983).

In conditions less severe than kwashiorkor albumin is also a gauge of nutritional status. An estimated 30% of patients entering hospitals are malnourished; efforts are being made to identify these patients so they can be given prompt nutritional therapy before surgical procedures are undertaken. United States Medicare diagnosis of malnutrition uses two of the following typical parameters: albumin concentration, total lymphocyte count, percent ideal body weight, and percent weight loss (Micozzi *et al.*, 1989). Of these and other measurements, such as skin-fold thickness and arm circumference, a low albumin level is the

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indicator most commonly associated with a longer hospital stay (Anderson and Wochos, 1982).

Owing to its relatively long 19-day half-life in plasma and to the compensatory factors mentioned above, the level of albumin is an "insensitive marker" of rapid changes in nutritional status (Rosse and Shizgal, 1980). It rises and falls only slowly, and so is a better reflector of the chronic state of nutrition than of recent changes. Replenishment of malnourished infants, for instance, is rapidly evident in the levels of transthyretin and fibronectin, plasma proteins with half-life of 1–3 days, but the albumin level rises only after 3 weeks or more (Yoder *et al.*, 1987). In malnourished adults on parenteral nutritional care albumin levels rise about 1 g/L/week (Tuten *et al.*, 1985).

2. *Renal Disease*

The renal glomeruli become slightly more permeable as diabetes mellitus progresses; this leakiness leads to slight increases in urinary albumin excretion, which are of interest in following the course of the diabetes, as discussed in Section II,B,3. Severe leakiness of the glomerular filtration mechanism marks the nephrotic syndrome; massive losses of plasma proteins occur, particularly of proteins <78 kDa in size. With decreasing glomerular filtration rate (GFR), kidney function diminishes and uremia results, with buildup of toxic metaholites that affect liver function and ligand binding by the albumin molecule.

a. Nephrotic Syndrome. In the nephrotic syndrome the daily urinary loss of albumin typically rises from ≈10 mg to 1–8 g, but can be as high as 17 g. Increased catabolism within the renal tubules (Kaysen and al Bander, 1990) adds to the albumin loss, so that the half-life of albumin in the plasma drops from 19 to ≈4 days (Jensen *et al.*, 1967). The liver is unable to match this demand and the circulating albumin concentration drops to 7–25 g/L; edema is common. Metcoff and Janeway (1961) showed that infusion of albumin in massive doses, 500 g in 22 days, could cure the edema temporarily, but the urinary loss climbed to 35 g/day.

In only 30–50% of patients does the liver respond with albumin synthesis above its normal rate of 13–14 g/day; the increases are typically 30–40% but can go to as high as 27 g/day, twice the normal value (Kaysen *et al.*, 1987; Ballmer *et al.*, 1992). In keeping with the dependence of albumin biosynthesis on the amino acid supply, a high-protein intake is needed for this response.

Hyperlipidemia is characteristic of the nephrotic syndrome, and parallels the amount of albumin lost. The hyperlipidemia is attributable to the large size of the lipoproteins, which prevents their renal loss, and also to a stimulus of lipoprotein production (Davies *et al.*, 1990). Whether the paucity of albumin as an acceptor of LCFAs from lipoprotein lipase action is a factor in the hyperlipi-

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demia has been conjectured but not established.

The albumin forms found in nephrotic urine are characteristically more acidic than those in normal urine, predominantly p*I* ≈4.7; this is the result of the high loading of LCFAs, which are in abundance, on albumin, which is in short supply (Ghiggeri *et al.*, 1987). An incidental finding in the nephrotic syndrome is a high proportion of albumin S–S-bonded dimers in urine samples that have been stored frozen (Birtwistle and Hardwicke, 1985). The same phenomenon can be observed with normal urine, but apparently the high protein concentration in the nephrotic syndrome together with the urea level in urine, 0.2–0.5 *M*, cause conformational changes which favor S–S bonding as the pH rises due to the loss of CO_2.

An experimental nephrosis bearing many of the features of the human nephrotic syndrome can be produced in rats by the injection for 7–8 days of the aminonucleoside of puromycin. Plasma albumin levels fall from 45 to 12 g/L., and extravascular and ascitic fluids accumulate; unlike the hypoalbuminemia of protein malnutrition, however, albumin is not drawn from the interstitial spaces into the circulation, the EV/IV ratio remaining about 1.7/1 (Sellers *et al.*, 1968).

The isolated rat liver responds to the hypoalbuminemia by accelerating albumin synthesis 40 and 60% on a per-gram liver basis as measured in the perfused liver (Lewandowski *et al.*, 1988) or in liver slices (Peters, 1973), respectively. The basis for the response is increased transcription of albumin mRNA (Yamauchi *et al.*, 1988), which rises 50–100% in concentration (Zähringer *et al.*, 1976). No consistent increase of hepatic albumin synthesis is seen *in vivo* (Peters and Peters, 1972), although it is observed in another experimental nephrosis, Masugi nephritis, evoked by injections of antikidney serum (Koerge and Oeff, 1963). A possible explanation is that the antiserum is specific for the kidney, whereas the puromycin drug depresses liver function as well as kidney function *in vivo*, but is washed out in the liver slice or liver perfusion systems.

b. Uremia. As the glomerular filtration rate falls and chronic renal failure sets in, proteins are no longer lost in large quantity in the urine, and albumin degradation in the proximal tubules subsides (Johansson *et al.*, 1977). A buildup of secreted toxins depresses albumin synthesis by the liver and there is an increase in intravascular and extravascular fluid volume, so the albumin level remains mildly subnormal, ≈35 g/L.

The rate of albumin synthesis measured in uremic patients *in vivo* is about 85% of normal (Bianchi *et al.*, 1978). In uremic rats protein synthesis by cell-free polysomes is decreased, and there appears to be an inability of polysomes synthesizing albumin to associate with the microsomal membrane (Grossman *et al.*, 1977). The concentration of albumin mRNA in the liver of uremic rats falls 45% (Yamauchi *et al.*, 1989), particularly in the membrane-bound polysomes. The responsible toxin(s) have not been identified, but they appear to act by

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destabilizing the albumin mRNA rather than by depressing its synthesis; the transcription rate is normal, and ribonuclease activity is increased in the hepatic cytoplasm (Zern *et al.*, 1984).

Toxic compounds affecting protein function, if not biosynthesis, have been widely sought in patients with renal failure. The review by Gulyassy and Depner (1983) describes this search. Two decades after studies suggested that binding capacity of serum proteins is affected (Breyer and Radcliff, 1954), Campion (1973) demonstrated a decrease in bound *o*-methyl red in 20 of 29 patients. The responsible agent was apparently tightly bound, because it could not be removed by dialysis. Because urea concentrations rise so markedly, carbamylation of amino groups by small amounts of cyanate produced from urea was first suspected. The level of carbamylation of albumin was found to be only 0.27 M/M, however, well below the level shown to affect warfarin binding (Bachmann *et al.*, 1981).

Many aromatic compounds were tested, leading to the discovery that indoxyl (Bowmer and Lindup, 1982) and furanoic (Mabuchi and Nakahashi, 1986) acids are highly active in depressing albumin-binding capacity for small anionic compounds. The most likely culprit is a furan dicarboxylic acid, 3-carboxyl-4-methyl-5-propyl-2-furanpropanoic acid (CMPF; Fig. 6-1), which shows a K_A of $4.6 \times 10^6 M^{-1}$ for a primary anionic ligand site of HSA (Henderson and Lindup, 1990). Because it displaces drugs such as furosemide and salicylate (Lim *et al.*, 1993), it apparently binds at Site I (see Table 3-5). Its high affinity for albumin explains its retention, and its increase in uremic plasma points to tubular secretion as its normal mode of excretion.

The binding at Site I of indicator dyes other than methyl red is also affected by CMPF in uremia. BCP (Mabuchi and Nakahashi, 1987) gives low values in inverse relation to the serum CMPF level when used to assay for albumin in the serum of patients with uremia. BCP (Wells *et al.*, 1985) and methyl orange (Dromgoole, 1973) are bound less strongly than usual in hemodialysis patients. In the latter case the cause was shown to be the marked increase in free LCFAs resulting from action of the heparin used in the procedure.

Therapy by hemodialysis has other effects on albumin. Cysteine is lost from

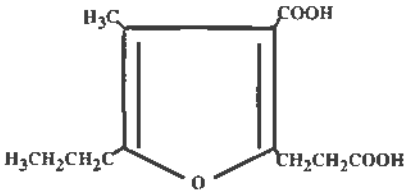


Fig. 6.1. Structure of 3-carboxyl-4-methyl-5-propyl-2-furanpropanoic acid, purported ligand-binding inhibitor found in uremic plasma (Mabuchi and Nakahashi, 1986).

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its mixed disulfide bond, so that the proportion of mercaptalbumin is higher after the treatment (Okajima *et al.*, 1985). "Middle molecule"-size peptides can be detected in the hemofiltrate (Kausler and Spiteller, 1991), including peptides 1–24, 1–26, 25–31, 330–333, and 407–423 from HSA; all of the cleavage sites are typical of peptic action. Such fragments are not found in normal plasma, and it would be interesting to know where they are produced and how they come to appear in the circulation in uremia.

In ambulatory patients receiving continuous peritoneal dialysis there is a significant risk of peritonitis. A decrease in circulating albumin level was shown to be one of the most critical prognostic signs pointing to a need for aggressive treatment (Murata *et al.*, 1993).

3. Diabetes

In diabetes the circulating albumin level is mildly depressed. A 30–40% fall in the rate of albumin synthesis in uncontrolled diabetic patients as well as in perfused livers of diabetic rats can be restored by *in vivo* insulin; one basis is a marked decline in transcription of albumin mRNA (Chapter 5, Section I,B,4,b). Albumin degradation and relative extravascular distribution volume are likewise decreased about 35% in diabetic rats (Murtiashaw *et al.*, 1983). The depression of metabolism extends to many other plasma proteins and does not appear to be specific for albumin (Jefferson *et al.*, 1983).

Other features of diabetes are the effects of nonenzymatic glycation of circulating albumin, the detection of a small increase in albumin urinary excretion as a harbinger of incipient kidney damage, and speculation that albumin from cow's milk may be an etiological factor in juvenile-onset diabetes.

a. Nonenzymatic Glycation. The spontaneously coupling of circulating glucose to proteins was first observed with hemoglobin; two laboratories almost concurrently reported nonenzymatic glycation of serum albumin (Dolhofer and Wieland, 1979; Guthrow *et al.*, 1979). The reaction can be demonstrated by long (7–28 days) incubation of albumin at 37° with high concentrations of glucose in the presence of antibiotics to inhibit bacterial growth, but whether the sites of coupling on the albumin molecule *in vitro* are identical to those *in vivo* is uncertain.

In the glycation reaction, an aldose such as D-glucose first forms an aldimine with an uncharged amino group of a protein via Schiff base formation (Fig. 6-2). In a second step an Amadori rearrangement causes the imine double bond to migrate down the carbohydrate chain to form a 2-keto derivative; in the case of glucose the result is a fructosamine derivative of the protein amino group. The sites of (*in vitro*) glycation of HSA are primarily Lys-525 (Shaklai *et al.*, 1984) plus contributions from Lys-199, Lys-281, Lys-439 (Iberg and Flückiger, 1986), and the α-NH_2 of Asp-1 (Robb *et al.*, 1989).

Glycated proteins can be measured by the thiobarbituric acid colorimetric

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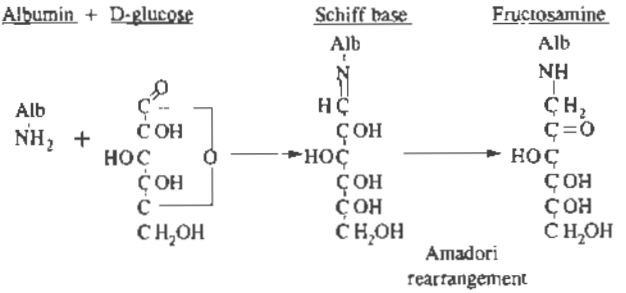


Fig. 6-2. Formation of glycated albumin.

assay for fructosamine (Guthrow *et al.*, 1979); because glycated albumin comprises only about half the total glycated plasma protein, the albumin should be isolated first. A more popular method is direct isolation of glycoalbumin by affinity chromatography on boronic acid–agarose columns (Rendell *et al.*, 1985), with the glycoalbumin being eluted by excess citrate at pH 4.5 and assayed by the BCG or other routine procedure for albumin. Results are expressed as percent glycoalbumin/total albumin, assuming 1 mol of sugar per molecule of glycoalbumin. The reference range in normal humans is 1–2%.

In an interspecies comparison of normal levels, glycated albumin was 2.4–3.2% in chicken, ducks, and turkeys, 2.0% in the rabbit, 1.5% in humans and the pig, 0.8% in the dog and the gerbil, and 0.5% and 0.3% in the rat and mouse, respectively (Rendell *et al.*, 1985). The normal relative glycation of hemoglobin is 2–11 times that of albumin in mammals, but only 0.2–0.3 times in birds; a reduced permeability of the avian red blood cell to glucose is postulated.

The degree of glycation of albumin is a function of the product of the plasma glucose concentration and the time of exposure. In diabetic humans whose glucose level is high, the relative glycoalbumin typically rises to 8% or more. The useful aspect of glycoalbumin to clinicians is its rapid turnover relative to that of glycohemoglobin. The albumin molecule has an average life of 27 days in the circulation compared to the life of a typical hemoglobin molecule of 120 days; there is some evidence that glycated albumin is degraded about 33% faster than unglycated albumin (Morris and Preddy, 1986; Kallner, 1990), which would accentuate the difference in turnover rates. Hence, although glycohemoglobin is the favorite test for following long-term control of blood sugar levels in diabetics, glycoalbumin is more useful in detecting changes in control over a period of 2–4 weeks. With worsening control significant changes in glycoalbumin are found even within 1 week (Winocour *et al.*, 1989).

If albumin half-life is slowed, as occurs in malnutrition and some other circumstances when the albumin level is low, a higher-than-expected degree of gly-

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cation is observed (Schleicher *et al.*, 1993).

Are there repercussions of *in vivo* glycation on the function of the albumin molecule? The question is unanswered as yet, most studies having been performed on albumin that was glycated *in vitro*. To date they show no effect of glycation on binding of LCFAs (Vorum *et al.*, 1991), of hemin (Shaklai *et al.*, 1984), or of Site-II drugs and tryptophan (Bohney and Feldhoff, 1992). If the primary site of glycation is indeed Lys-525, on the surface of the molecule, this should not be surprising. The study by Shaklai *et al.*, however, which used *in vivo*-glycated albumin, reported a decrease of 50% in affinity for bilirubin and of 95% for the highly unsaturated *cis*-parinaric acid, as well as evidence of a conformational change that reduced the quantum yield of tryptophan fluorescence by 30%.

Glycation produces an albumin molecule with one less positive charge, so its net charge is −16 compared to −15 (Table 2-3). This increased negativity is reported to promote more rapid uptake into isolated endothelial microvessels (Williams *et al.*, 1981), into the endoneurium of rat sciatic nerve (Patel *et al.*, 1991), or across renal glomerular basement membranes in an *in vitro* model (Daniels and Hauser, 1992). The transcapillary escape rate of glycoalbumin, however, is slightly reduced compared to that of unmodified albumin, either in normal or diabetic humans (Bent-Hansen *et al.*, 1993), an effect that is yet to be reconciled. The presence of an endothelial cell receptor specific for glycated albumin has been proposed (Wu and Cohen, 1994).

Antibodies to the *in vivo* form of glycoalbumin have been sought but not detected (Gregor *et al.*, 1986). If albumin is glycated in the presence of a reducing agent such as borohydride, so that the glucose becomes a glucitol–lysine adduct, antibodies to this group are claimed to be widely present in both normal and diabetic subjects, as both IgG and IgM isotypes (Nakayama *et al.*, 1985; Mangili *et al.*, 1988).

b. Increased Urinary Albumin. With the availability of sensitive immunochemical methods permitting measurement of albumin in urine in the range 5–30 mg/L (Keen and Chiouverakis, 1963), albumin has begun to supplant total protein of urine in detecting the onset of renal involvement in diabetes. A 1987 international symposium established the following classification: albumin excretion rate <20 μg/min (equivalent to ≈30 mg/day), normoalbuminuria; 20–200 μg/min, microalbuminuria; >200 μg/min, macroproteinuria (Hawthorne, 1989).

The importance of the microalbuminuria range is that it can predict future renal damage before other tests become positive and in time to take preventive measures. Of patients with urinary albumin excretion rates in the range 20–200 μg/min, 80% of those with Type-I (insulin-dependent) diabetes (IDDM) and 25% of those with Type-II (noninsulin-dependent) diabetes (NIDDM) will

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develop nephropathy within about 15 years. Because there are about 10 times as many Type-II patients as Type-I patients, more cases of potential renal damage are likely to be detected by testing of Type-II diabetics. This group has seven times the normal death rate (Mattock *et al.*, 1992), particularly from cardiovascular disease (Winocour *et al.*, 1992).

Therapy for Type-I diabetes consists of stricter than usual control of blood sugar and blood pressure levels. For Type-II patients weight control, reduction of dietary protein intake, reduction of plasma cholesterol concentration (Metcalf *et al.*, 1993), and other "heart-healthy" steps are recommended.

Choice of the term microalbuminuria was intended to denote a slight increase in excretion of albumin. It was an unfortunate choice, because biochemists use "micro-" and "macro-" prefixes to refer to proteins of abnormally low or high molecular mass, e.g., β-microglobulin, which is a fraction of the size of immunoglobulin G, or macroamylase, which is a complex of amylase with antibody. The urinary albumin in microalbuminuria is of normal size, and the condition is actually a "minimal elevation of urinary albumin." But this label is verbose and the catchier phrase appears already embedded in medical jargon.

The mechanism of the increased albumin loss is believed to involve glycation of the glomerular basement membrane. This filter membrane has an effective pore radius of 55 Å, large enough to pass the albumin molecule with some frequency, but actually allows only 0.004% of the perfusing albumin to leak through the filter. A strong negative charge on the basement membrane, chiefly from sulfate ions of glycosaminoglycans, adds selectivity in the rejection of the highly anionic albumin molecule; by contrast, hemoglobin, which has the same molecular weight, is filtered to the extent of 3%.

If glycation due to diabetes interferes with glycosaminoglycan production in the basement membrane, causing it to lose some of its negative charge, the membrane becomes less effective in retaining albumin (Bent-Hansen *et al.*, 1993). Commonly in diabetics there is also an increase in the glomerular filtration rate, partly due to an increase in blood pressure above 140/90 mm Hg (Gosling and Beevers, 1989). The net result is an increase in filtered albumin. The proximal tubule cells, which normally take up and degrade 99% of the filtered albumin (Chapter 5, Section III), increase their uptake as the filtered albumin load increases, so that still only milligram amounts of albumin appear in the urine.

With increasing renal involvement, both size selectivity and charge selectivity of the glomerular filter deteriorate (Viberti and Keen, 1984), and plasma proteins pass through in greater amounts. The ability of the proximal tubule cells to capture and degrade these proteins becomes overwhelmed. In the late stages GFR decreases, reducing the amount of protein loss but leading to renal failure.

The behavior of glycated albumin in this progression is complex. Its slightly greater negative charge causes it to be filtered slightly less well than unmodified

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albumin through the negatively charged basement membrane. The receptors on the proximal tubule cells, however, are even more discriminating against binding negatively charged forms, and so degrade less glycoalbumin than they do albumin. The result is that the proportion of glycoalbumin in normal urine is 10% compared to the 1.5% found in normal plasma (Cha *et al.*, 1991).

With progression of diabetic nephropathy, both forms pass through the glomerular filter in greater quantity, and the proximal tubule uptake mechanism becomes inundated so that neither is effectively salvaged. Both forms then reach the urine in larger amounts, and the proportion of glycoalbumin in urine again approaches that in plasma.

Analysis of the protein forms in urine with diabetic nephropathy by isoelectric focusing shows a variety of abnormally acidic glycated species, with p*I* as low as 4.7. These are apparently multiglycated forms of albumin (Ghiggeri *et al.*, 1985). Also observed are some highly acidic forms caused by binding of high loads of LCFAs, as in the nephrotic syndrome (Hayashi *et al.*, 1990).

c. BSA as Cause of Juvenile Diabetes. Links between consumption of cow's milk in infancy and development of juvenile IDDM have appeared frequently. The disease occurs particularly in families carrying a susceptibility gene in the major histocompatibility locus MHC class II, DR and DQ. In isolated places, such as Samoa, where infants are breast-fed, IDDM does not occur. Diabetes-prone strains of rats or mice reared for the first few weeks of life without exposure to cow's milk products do not develop the disease (Martin *et al.*, 1991).

Although serum albumin is only present in cow's milk at 0.4 g/L, it appears to be antigenic in man as a food allergen. BSA is found in circulating immune complexes in patients with IgA nephropathy (Yap *et al.*, 1987) or recurrent angioedema (Lefvert, 1993), and as an IgE complex in eosinophilic gastroenteritis (Verdaguer *et al.*, 1993).

A segment of BSA, residues 126–144 in the ascending arm of loop 3, with the sequence FKADEKKFWGKYLYEIARR, termed "ABBOS" by the group at Toronto (Martin *et al.*, 1991), was found by them to show homology with residues ≈56–76 of a cell-surface protein, p69, of the pancreatic islet β-cell, and has been synthesized by them as a test antigen. (Note: I have changed the sequence numbering given by them for this segment to agree with the actual BSA sequence; their peptide also shows Asn instead of Trp at position 134.) They find circulating humoral antibodies against this peptide in children newly diagnosed with IDDM; these antibodies disappear along with the disappearance of β-cell function after a few years.

Their hypothesis (Karjalainen *et al.*, 1992) is that this peptide reaches the immune system of infants receiving cow's milk during the first 3 months of life, when the gut allows large fragments of proteins to be absorbed. In the case of susceptible infants, the peptide shares an epitope with the β-cell p69 protein, and

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evokes an immune response to this protein that persists even after the gut matures. Later, unrelated infections or other acute-phase reactions induce the expression of p69 on the surface of β-cells, and the immune system attacks and destroys these cells; insulin production is simultaneously lost and IDDM results.

Although this is an attractive scheme that explains many phenomena about the onset of a severe disease, Atkinson *et al.* (1993) note that cellular, not humoral, immune mechanisms are considered to cause IDDM. They could not demonstrate a proliferative response of mononuclear cells to BSA or to the above peptide. Their results question the relevance of BSA or its fragments in the pathogenesis of the disease, and favor the role of autoimmunity.

4. Gastrointestinal Pathology; Liver Disease

a. Liver Disease. In most patients with obstructive or viral hepatitis, or even cirrhosis, the albumin level is near 30 g/L; with toxic hepatitis or hepatic tumors it drops to the range 25 g/L (Skrede *et al.*, 1975). However, although albumin is made by the liver, its concentration in plasma is a poor gauge of the severity of liver disease.

The rate of albumin synthesis is variable in the presence of hepatic cirrhosis, and in many cases remains normal, whether studied *in vitro* (Huberman and Soberon, 1970) or *in vivo* (Rothschild *et al.*, 1972b; Ballmer *et al.*, 1993). The concentration of albumin mRNA in the liver likewise did not correlate well with the severity of cirrhosis (Ozaki *et al.*, 1991); it does fall with viral infection (Annoni *et al.*, 1990).

Qualitatively, the albumin band of patients with liver disease often appears broader than normal on electrophoresis, extending forward in the anodic direction (Laurell, 1972). This phenomenon is characteristic of severe jaundice, when the albumin molecule becomes more negative owing to tightly bound bilirubin with its two carboxylate groups, or to covalently coupled δ-bilirubin, which blocks an ε-amino group (Chapter 3, Section I,B,2). δ-Bilirubin has actually been found to be a favorable sign of acceptance of liver transplants, apparently as a measure of the conjugating ability of the new liver; if the δ-bilirubin was less than 30% of the total bilirubin, rejection of the transplant was imminent (Wu *et al.*, 1990).

The effects of alcohol intake, both chronic and acute, on albumin synthesis have been studied for many years by M.A. Rothschild and the group at the New York Medical Center (Rothschild *et al.*, 1983). The acute effect, ≈4 h after administration of alcohol to rats, is to hinder albumin secretion, with accumulation in the Golgi apparatus (Volentine *et al.*, 1986), possibly through a direct effect of alcohol on the cytoplasmic membranes. Alcohol causes a disaggregation of polysomes in the perfused rabbit liver, which is similar to that seen in fasting, and, indeed, the effect of alcohol on albumin synthesis appears to be largely a

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nutritional one—if an amino acid mixture is given together with the alcohol there is no depressant effect (Jeejeebhoy *et al.*, 1972b). Even tryptophan plus the basic compounds, arginine and spermine, by themselves can suffice to override the effect of alcohol. The positive effect of spermine on assembly of polysomes by aiding mRNA–ribosome attachment was mentioned in Chapter 5 (Section I.C.3).

Acetaldehyde, the initial product of alcohol oxidation in the liver, will couple covalently to protein side chains and has been suspected of causing a toxic inhibition of albumin synthesis. Addition of acetaldehyde to the perfusate of rabbit livers depressed albumin synthesis about 40%, but its action did not resemble that of alcohol, in that the polysomes remained aggregated and the effect was seen only in fed, not fasted, animals (Oratz *et al.*, 1978).

The rat can regenerate its liver nearly completely within 24 h of surgical removal of two-thirds of the liver mass. In this popular experimental system albumin production per unit liver mass initially falls, driven by a reduction in transcription rate that resembles the acute-phase reaction (Kreig *et al.*, 1980; Milland *et al.*, 1990). Again the limiting factor in albumin synthesis appears to be nutrition, however. Provision of ample amino acids (10× normal plasma levels) in the perfusate of regenerating rat liver restored albumin synthesis to normal (Lloyd *et al.*, 1975).

Direct liver damage by carbon tetrachloride reduces albumin synthesis severely and abruptly whether measured *in vivo* (Smuckler, 1966), in liver slices (Peters, 1973), or in the perfused liver (Rothschild *et al.*, 1972a). The acute effect appears to be a dissolution of the hepatocellular membranes. By 24 h albumin gene transcription falls 85% in the rat, whereas AFP gene transcription increases markedly to 50% of the normal level for albumin (Panduro *et al.*, 1988). The liver then begins regenerating, and by 3 weeks the albumin and AFP transcription rates return to normal.

Ascites is a common feature of alcoholic cirrhosis. It is primarily the result of portal hypertension rather than hypoalbuminemia, because circulating albumin levels as low as 20 g/L do not produce ascites. An important clinical question is the differentiation of ascites fluid due to uncomplicated portal hypertension (a transudate) from that arising from peritonitis or malignancy (an exudate). The albumin level is typically higher in an exudate, and the older criterion to classify a fluid as an exudate if it contained >25 g/L total protein has now been updated by the use of the gradient between the plasma and ascites albumin levels; uncomplicated ascitic fluid shows an albumin level >11 g/L lower than that of the plasma level, whereas infectious or malignant ascitic fluid has a smaller gradient (Parë *et al.*, 1983). Numerous clinical reports have confirmed this plasma/ascites albumin gradient of 11 g/L as an effective cutoff; one report suggested that 12 g/L would be better (Roth *et al.*, 1990), and another proposed a small correction for serum globulin concentration if the gradient value is near the cutoff level (Hoefs, 1992).

An association of albumin with hepatitis B viral (HBV) infections has been

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proposed following observation that the HBV would bind to polymerized albumin from species susceptible to hepatitis (Imai *et al.*, 1979) and that the polymerized albumin likewise bound to hepatic cell membranes (Lenkei *et al.*, 1977). The polyalbumin binds either to the viral core, the Dane particle, or to the 20-nm spheres of its surface antigen, in the pre-S2 region (Pontisso *et al.*, 1989; Krone *et al.*, 1990). Doubt has been raised about the significance of this binding, however, because it seems to require unnatural albumin polymers produced by coupling with glutaraldehyde or transglutaminase (Thung *et al.*, 1989), and is not seen with albumin polymers isolated from commercial preparations (Wright *et al.*, 1987) or with carbodiimide- or heat-induced polymers (Yu *et al.*, 1985). Glutaraldehyde-polymerized HSA is taken up primarily by scavenger (endothelial or Kupffer) cells in perfused liver and is rapidly degraded, so that virus particles bound to it would not effectively reach parenchymal cells (Wright *et al.*, 1988).

Autoantibodies to normal human albumin are frequently observed in liver disease (Tamura *et al.*, 1982). Usually of IgA class, they are seen most often with Laennec's cirrhosis (40% of cases studied) and chronic active hepatitis. It is not clear why this prevalent extracellular protein becomes antigenic; because IgA antibodies are associated with secretions, Hauptmann and Tomasi (1974) suggested that the autoantibodies form against albumin altered during metabolism in the GI tract. In patients infected with HBV, spontaneous secretion of IgG antiHSA antibodies has been reported (Hellström and Sylvan, 1989). Their production was shown to be regulated by T lymphocytes sensitized to HBV surface antigen plus HSA; their relation to the HBV–polyHSA association cited above is not clear.

b. Diseases of GI Tract. Three causes of transient bisalbuminemias, in contrast to the permanent, genetic bisalbuminemias discussed in Chapter 4 (Section IV), are covalent binding of drugs, particularly β-lactams, premature leakage of proalbumin from an inflamed liver (Chapter 5, Section I.D.4), and proteolytic cleavage in pancreatic disease. Porta *et al.* (1980) reviewed 5 cases of bisalbuminemia probably due to pancreatic disease and 57 cases due to drugs in the period 1971–1979. The pancreatic form is usually faster moving on electrophoresis; it is due to cleavage of circulating albumin by escaped pancreatic enzymes, such as chymotrypsin, elastase, and carboxypeptidases A and B (Rousseaux *et al.*, 1976). In most cases a fistula can be found from the pancreas to the peritoneum or the pleural space, with ascites or pleural fluid present in which the double albumin is more prominent than in plasma. Surgical closure of the pancreatic fistula leads to disappearance of the abnormal albumin band within hours (Albaret *et al.*, 1981; Keidar *et al.*, 1986).

In hemorrhagic pancreatitis large quantities of hematin are released, overloading the normal carrier protein, hemopexin, and forming methemalbumin. Less often diagnostically used now than it was earlier, the hematin attached to methemalbumin could be detected by a simple guaiac or benzidine test and was a helpful diagnostic aid in acute abdominal emergencies (Goodhead, 1970;

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Kiekens *et al.*, 1971).

In the intestines, malabsorption from mucosal inflammatory diseases or lymphatic obstruction will hinder amino acid absorption and produce hypoalbuminemia on a nutritional basis. Overproduction of ammonia from amino acids by bacterial overgrowth has been reported to preempt essential amino acids (arginine, ornithine) to form urea at the expense of forming albumin (Rosenoer *et al.*, 1977). Direct loss of albumin into the gut normally amounts only to about 1 g/day, but in protein-losing enteropathies loss can reach 36 g/day and result in compensatory albumin synthesis at the highest rates known (Table 5-3).

Whether hypoalbuminemia can be a cause of intestinal pathology as well as its result is still a matter of debate. Albumin levels below 20 g/L are associated with diffuse intestinal edema, demonstrable radiologically (Granger and Barrowman, 1983). In turn, the edema may lead to diarrhea (Schwartz and Darrow, 1988), which further aggravates the hypoalbuminemia by depressing the supply of amino acids for albumin biosynthesis. The need for more data to support this connection has been stressed (Mobarhan, 1988); however, in a study of 35 critically ill patients, diarrhea developed in every patient with a serum albumin level <26 g/L, whereas it developed in none with a level ≥26 g/L (Brinson and Kolts, 1987). In a larger study (Hwang *et al.*, 1994), nearly three times as many patients with albumin levels below 20 g/L developed diarrhea as did those with higher levels; the incidence of diarrhea was significantly higher in those whose hypoalbuminemia was chronic—due to malnutrition—rather than acute, such as following severe burns.

Measurement of albumin in feces has been proposed as a substitute for fecal hemoglobin (occult blood) in the diagnosis of occult intestinal bleeding, but α_1-antitrypsin was favored for its greater resistance to intestinal proteolysis (Morrow *et al.*, 1990). This very lability to digestion in the upper intestine, however, has prompted interest in fecal albumin as a more specific test for hemorrhage occurring in the colon, where proteolysis is minimal (Kutter *et al.*, 1991). An electroimmunoprecipitation assay, senstive to 2 μg/mL (Otto and Nemeth, 1993), has been proposed for determining both fecal albumin and hemoglobin simultaneously.

5. Acute-Phase Reaction; Immune Disorders; Cancer

a. Acute-Phase Reaction. When the body mounts an acute-phase reaction in response to any of a number of stressful situations, albumin concentration in plasma characteristically falls. The stress may be infection, inflammation, a myocardial infarction, surgery, a burn, or even repeated injections of phenobarbital (Bertaux *et al.*, 1991). The average fall in albumin level following surgery is 5 g/L (Doweiko and Nompleggi, 1991a), or about 15% after 3 days (Aronsen *et al.*, 1972).

One factor in the decline is an increase in capillary permeability, with a shift

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of albumin from the intra- to the extravascular spaces. The resulting interstitial edema is detrimental to wound healing; accumulation of albumin in wounds is 10-fold that in normal tissues (Mouridsen, 1969). In burns, particularly, the albumin normally stored in skin (Table 5-4) is partially lost, and the compromised lymph return causes accumulation and loss of albumin through one-way transport into weeping wounds (Tögl Leimüller *et al.*, 1986).

Albumin synthesis falls some 60% in rats 24 h after creation of a turpentine abscess (Schreiber *et al.*, 1982); production of other negative acute-phase proteins such as transferrin and transthyretin falls even more. As discussed in Chapter 5 (Section I.B.4.a), the cause is depression of albumin gene transcription effected primarily by the cytokine, IL-6. Other cytokines earlier in the reaction pathway, IL-1 and GM-CSF, appear to act less directly. The IL-6 that affects the liver may arise primarily from Kupffer cells (Kowalski-Saunders *et al.*, 1992). A restorative effect is the accompanying release of adrenocortical hormones, which in turn promote release of free amino acids by muscle breakdown and assist albumin synthesis. Yet prior administration of prednisolone, before the turpentine, does not prevent the fall in circulating albumin level (Ballmer and Studer, 1994). Albumin itself may contribute to the damage in the acute-phase situation, being degraded by macrophage enzymes to produce peptide 409–423, which has a histamine-releasing action (Chapter 2, Section I.D).

b. Immune Disorders. Anti-HSA antibodies have been observed in diabetes (Eilat *et al.*, 1981), in 5% of diabetic patients in one study, a fivefold greater occurrence than in nondiabetic persons (Gregor *et al.*, 1986). Sera from patients with familial dysautonomia contain high levels of anti-HSA antibodies, which correlate with levels of anti-BSA antibodies and decline exponentially after infancy (Chapman *et al.*, 1993), suggesting that they contribute to the pathological changes in this condition.

Other albumin-related antibodies are seen in drug allergies. Drug–albumin complexes can be identified by the "tailing albumin" phenomenon on electrophoresis of sera from patients who received prolonged treatment with nitrofurantoin (Wager and Teppo, 1978) or by the more rapidly moving albumin seen with penicillin allergy (Lafaye and Lapresle, 1988a). In these cases the drug has apparently acted as a hapten after covalently coupling to HSA (see Chapter 3, Section I.C.3). Occupational asthma resulting from nickel binding is well recognized. It has been traced to antibodies against nickel(II) specifically bound to the amino-terminal tripeptide sequence; the antibodies can be blocked by Ni-Gly-Gly-L-His (Nieboer *et al.*, 1984).

Albumin that is shed with flakes of skin can evoke allergies. Rat or mouse SA is recognized as an airborne agent causing anaphylactic reactions among animal handlers (Wahn *et al.*, 1980; Sakaguchi *et al.*, 1989). Even human albumin has

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been identified as an allergen in house dust (Witteman *et al.*, 1993). Among causes of food allergy, BSA is a possible cause of Type-I diabetes (see Section II,B,3,c). Chicken serum albumin (α-livetin) occurring in egg yolk is believed to be responsible for "bird-egg syndrome," a condition distinct from egg white allergy (Szepfalusi *et al.*, 1994).

In rheumatic diseases albumin levels in serum tend to be low, correlating with the occurrence of juxtaarticular erosions or peptic ulcer (Niwa *et al.*, 1990). The level is generally lower in rheumatoid arthritis than in systemic lupus erythematosus, and is related to accelerated albumin catabolism. If the albumin gains access to normally protected areas, however, it can contribute to inflammation. Along with IgG, albumin has been identified, bound by S–S bonds to keratan sulfate-rich proteoglycans, in the articular cartilage of patients with arthritis (Mannik and Person, 1993).

A highly acidic nine-amino acid antigenic unit on the malaria parasite, *Plasmodium falciparum*, antigen 11.1, will generate antibodies that also react with human albumin (Mercereau-Puijalon *et al.*, 1992). The peptide has the sequence PEELVEEVL and the responsible site in HSA is believed to be residues 181–189, LDELRGEDK, with weak homology but in a region where HSA differs from the albumins of other species (Fig. 4-3). It does not coincide with any of the proposed antigenic sites of Atassi (Chapter 3, Section III,B). The cross-reaction may not be merely a chance phenomenon, but a device to assist the parasite to escape immune countermeasures in a manner reminiscent of the binding of albumin by streptococcal protein G (Chapter 3, Section I,E,6).

c. Cancer. The marked depression of the circulating albumin level in the presence of progressive malignancy has been recognized since at least 1950 (Mider *et al.*, 1950). Hypoalbuminemia as a prognostic aid in various common types of cancer was described in Section II,A; in several clinical studies it was the most significant single marker.

Thought to be tied to anorexia, the low albumin level cannot be raised toward normal even by parenteral nutrition strenuous enough to cause a gain in total body weight (Gray and Meguid, 1990). The primary cause of hypoalbuminemia is a specific inhibition of albumin gene transcription by the tumor necrosis factor, TNFα, reducing the level of albumin mRNA as much as 90% (Chapter 5, Section I,B,4,a). Injection of TNFα also has the effect of increasing transendothelial passage of albumin (Hennig *et al.*, 1988), which acts to lower its concentration in the circulation.

Another factor is the increased albumin degradation observed with malignancy (Rossing, 1968). The half-life of rat ^{35}S-labeled albumin was shortened by 33% in rats bearing the Walker 26 carcinoma or the 2056 sarcoma (Hradec, 1958). Tumors utilize albumin and other plasma proteins for their nutrition at a greater rate than do normal tissues; the uptake of labeled albumin was threefold

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higher in sarcomas than in mouse liver (C. Andersson *et al.*, 1991). The increased breakdown is largely due to host factors and is not caused by abnormalities in the albumin molecule, which appeared normal in these mice (Andersson *et al.*, 1990).

When hepatocytes become malignant there is a graded loss of albumin-synthesizing ability depending on the degree of dedifferentiation of the tumor. Four subclones of the rat hepatoma cell line FU5-5, for instance, produce albumin at the rate of 0, 0.3, 1.7, and 16 μg/mg cell protein/48 h (Wolf *et al.*, 1983). The Morris hepatomas 7777 and 7800 synthesize rat albumin about 9% as rapidly as does normal rat liver as judged by incorporation of labeled leucine, but the newly formed albumin is not secreted and is apparently digested within the cell (Ove *et al.*, 1972). Lack of albumin production and a switch to AFP production are used as markers of the degree of deviation of hepatomas from normal hepatic phenotype. Metastases of primary hepatomas as blood-borne hepatic tumor cells are now readily detected through PCR analysis of the blood cellular fraction for albumin mRNA (Hillaire *et al.*, 1994).

Paraproteins released by plasma cell tumors and lymphomas can display unusual properties. A rare HSA–monoclonal IgG complex in a patient with Von Willebrands disease harbored low-affinity binding sites for bilirubin (Gulian *et al.*, 1993). A monoclonal IgM protein binds bromcresol green and interferes with the commonly used BCG assay (Reed, 1987). In five reported cases monoclonal proteins produced by myelomas had anti-HSA immune specificity, presumably a random occurrence. Two were of IgA and three of IgG class, detectable as circulating antigen–antibody complexes; one of the IgA complexes was attached to HSA by S–S bonds (Eilat *et al.*, 1981). Two of the HSA–IgG complexes acted as cryoglobulins, precipitating in the cold, and could readily be crystallized (Jentoft *et al.*, 1987). They contained HSA/IgG-lambda in the ratio 2:1, and sedimented at 12.5S. The antigenic site on the HSA was believed to be calcium dependent because citrate prevented complex formation.

6. *The Neonate; Aging*

a. The Neonate. The rise in serum albumin level from low values in the fetus to nearly normal ones at birth is shown in Table 6-1. A study with [^{15}N]glycine in premature infants found that their low albumin level is not the result of an inadequate synthesis rate, which was 500 mg/kg/day (compare Table 5-3), but of high turnover, the half-life of their albumin being ≈6 days, threefold the adult rate (Yudkoff *et al.*, 1987). Liver slices of neonatal rats produce albumin at a rate increasing with gestational age to slightly more than twice the adult rate by day 15, then gradually declining (Wise and Oliver, 1967).

For several years there was conjecture that the albumin of newborns was

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different from that of adults, in the manner in which fetal hemoglobin differs from the adult form. Chiefly this concern was raised because the binding capacity for bilirubin was found to be only about 0.6 that of the albumin of adults, and BCG, warfarin, and certain sulfa drugs showed lower binding capacities as well. The interfering effect is also seen in the maternal circulation (Ritter *et al.*, 1985). Isoelectric focusing of cord blood sera showed the albumin to have a separate, faster component (Gallango *et al.*, 1981), but on isolation no differences were found in size, amino acid composition, amino-terminal sequence, or immunological properties (Gitzelmann Cumarasamy *et al.*, 1979). Spectral properties indicated a tertiary structure similar to that of adult albumin (Jori *et al.*, 1988).

This riddle was largely resolved when it was found that treatment of cord blood albumin with charcoal at pH 2.75 restores the binding capacity for bilirubin and the other compounds to normal (Huntley *et al.*, 1977). As in uremic sera, there is no abnormality of the albumin molecule, but a strongly bound competing ligand that blocks the site for bilirubin. It apparently competes only at Site I, because the binding of diazepam is normal in cord blood (Brodersen and Honoré, 1989). High levels of free fatty acids have been proposed (Ostrea *et al.*, 1983), but the $\bar{\nu}$ would have to exceed 6 LCFA/SA to displace bilirubin. By about 3 months of age the binding characteristics of neonatal albumin have returned to normal (Ritter and Kenny, 1986).

One candidate ligand is 2-hydroxybenzoylglycine (Suh *et al.*, 1987), present in both fetal and maternal circulations; although only found in ≈3 μ*M* concentrations, it displaces bound bilirubin and arachidonate of serum from ≈60 down to 9 μ*M* in *in vitro* experiments. Its source is unclear, but it is a conjugate of salicylic acid and glycine, and a relative of hippuric acid. The ligand demonstrated to block Site I in uremia, CMPF (Fig. 6-1), is another candidate without substantiation.

An inability to sequester bilirubin by the newborn is particularly crucial considering the danger of damage to the brain from kernicterus (Brodersen and Stern, 1990). Whereas the primary bilirubin site of albumin of a neonate would carry 0.52 m*M* bilirubin, equivalent to 30 mg/dL, the competing ligand lowers this "safe" level to about 20 mg/dL. Competition for the primary bilirubin site by numerous drugs adds a complicating factor (Robertson *et al.*, 1991).

The need to quantitate the ability to sequester bilirubin led to the development of various methods to measure the "reserve bilirubin binding capacity" or the "reserve albumin concentration" (Cashore *et al.*, 1983) of serum. Basically these techniques measure the available capacity by addition of a bilirubin load followed by removal of that bilirubin that is in excess of the capacity of the primary site, using Sephadex or HPLC; alternatively they measure the capacity to bind the purported bilirubin analog, MMADS (Francoual *et al.*, 1990). Comparing the rate of rise of the serum bilirubin with the measured reserve capacity allows neonatologists to judge when therapeutic measures need to be instigated.

Therapy customarily includes photoisomerization of bilirubin in the skin by

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light in the range 400–500 nm, where the bilirubin molecule absorbs. Exchange transfusions with whole blood in addition effectively increase the albumin reserve; infusion of HSA increased the fall in circulating bilirubin concentration in a recent study (Caldera *et al.*, 1991), but may not improve the amount of reserve albumin owing to the displacing effect on bilirubin of stabilizers added to commercial albumin in pasteurization (Robertson *et al.*, 1991).

Paradoxically, the elevation of serum bilirubin in the neonate may have a benevolent function. The rise in serum bilirubin in the first 2 days of life was lower by half in infants with illnesses considered to enhance free radical production than in healthy infant controls (Benaron and Bowen, 1991). The missing bilirubin was proposed to have been consumed as an *in vivo* antioxidant (see also Chapter 5, Section II,B,3,b).

b. Aging. The slight decrease in plasma albumin level with increasing age was described in Section II,A and Table 6-1. The accompanying decrease in albumin synthesis rate (Table 5-3) may be largely the result of less effective nutrition, but diminished transcription of the albumin gene (Singh *et al.*, 1990) and alterations in the nucleotide sequence of the cytoplasmic mRNA, making it less active (Horbach *et al.*, 1984), are contributing factors.

Modifications to the albumin molecule are mainly the result of accumulated changes occurring in the circulation with time (Chapter 5, Section II,D); the slower turnover allows more opportunity for such changes to occur in the elderly. Decreased drug binding was evident as a doubling of free concentrations of salicylate and diazepam in a comparison of healthy young versus aged subjects (Viani *et al.*, 1991). Tollefsbol and Cohen (1986) have reviewed the possible role of the collected aberrations in proteins as causes of the physiologic decline associated with aging; the correlation remains just an interesting possibility.

7. Drug Binding

Because it is the free form of a drug that is active, and also that is metabolized, the extent of binding to a protein controls both its effect and the duration of the effect. Clinical pharmacists are well aware that decreased binding of a drug to albumin can cause toxicity owing to an increase in the concentration of its free form. Competition of drugs that share the same site in albumin is a frequent cause of toxicity. A depressed binding can also result from interfering endogenous compounds, such as CMPF found in uremia and perhaps in the newborn (Section II,B,2,b), from covalent modification of albumin by other drugs such as aspirin or penicillin (Chapter 3, Section I,C,3), or simply from a deficit in albumin itself (hypoalbuminemia).

Examples are the gradual loss of consciousness in hypoalbuminemic

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patients receiving phenytoin (Lindow and Wijdicks, 1994), and hemorrhaging due to the increased action of warfarin when phenylbutazone is given. Adverse reactions to diazepam tripled in patients with albumin concentrations less than 30 g/L compared to those with normal albumin levels (Vallner, 1977). Drugs that are particularly prone to show clinically important effects are those that are more than 90% bound to albumin and that have relatively narrow ranges of therapeutic concentrations. The work by Koch-Weser and Sellers (1976) is recommended for an overview of the role of albumin in pharmacokinetics. Other reviews cited in Chapter 3 (Section I,C) are useful for further examples of interactions.

III. PARENTERAL USES

The human albumin product that was developed for the treatment of shock on the battlefields of World War II (Chapter 1) was quickly adopted by surgeons in civilian hospitals after peace was restored. HSA Fraction V, USP, was, and still is, an attractive agent for correction of hypovolemia. Required by the Food and Drug Administration to contain ≤4% globulins, it usually contains <2%. It is packaged as normal serum albumin (NSA) in 5 or 25% (w/v) solution containing <160 m*M* Na^+ and 0.08 mmol sodium acetyl-L-tryptophanate plus 0.08 mmol sodium caprylate per g albumin, equal to 5.4 M/M of each, and is stable for 5 years at 2–10° C. A unit of albumin is 12.5 g, equivalent to 250 ml of the 5% or 50 ml of the 25% solution; the 25% solution is also termed "salt-poor albumin" because it contains less sodium per gram of albumin than does the more dilute preparation, and is useful when volume expansion is desired without increasing the sodium load, as in the nephrotic syndrome or hepatic failure. Donors are screened for hepatitis and HIV infection, and all units are pasteurized by heating at 60 ± 0.5° for 10 h in their final containers, so NSA is free from concern of transmission of viral disease; nor is cross-matching required as it is with whole blood or red cell infusions.

The problem with NSA is that it has become *too* attractive as a therapeutic agent. From the ≈15,000 kg produced during World War II the output of therapeutic NSA rose to ≈76,000 kg in 1974 (Tullis, 1977b), to 250,000 kg in 1984 (Overby, 1985), and to 311,000 kg with a market value of US $937 million in 1990 and of $1.1 billion in 1992 (Reasor, 1994). Although it seldom causes harm, it is an expensive drug, $30–60 for a unit, which can consume 30% of a hospital pharmacy's supply budget (Boutros, 1986). Moreover, much of the use has been felt to be inappropriate, 60% in 1979 (Alexander *et al.*, 1979) and even higher, 74%, a decade later (Stumpf *et al.*, 1991), despite repeated cautions by reviewers to use albumin wisely.

Reviews published on the parenteral use of albumin include those by Tullis (1977a, b), Alexander *et al.* (1982), Hastings and Wolf (1992), and a particularly comprehensive one by Erstad *et al.* (1991). Several conferences have been con-

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vened to consider recommendations for appropriate use, among them a Workshop at the National Institutes of Health in 1975 (Sgouris and Rene, 1975) and a later one at Aspen, Colorado, in 1988 (Andrassy and Durr, 1988). The consensus of these conferences and of critical reviews is that administration of HSA can be justified for acute circulatory problems caused by hypovolemia, but it is seldom warranted merely to raise the albumin concentration in hypoalbuminemia.

A. Circulatory Support

The accepted indications for use of intravenous albumin are listed in Table 6-2. Only shock due to recent blood loss is included, because shock incident to acute-phase or hyperallergic reactions is accompanied by increased albumin flux to the extravascular compartment, and adding to the albumin pool in this case would only expand this compartment and bring on edema. The lung is particularly susceptible to albumin overloading, and albumin administration for respiratory distress has been a matter of debate because pulmonary edema may result (Doweiko and Nompleggi, 1991c). Cardiac failure is another hazard of overzealous increase in the circulatory volume.

Other criteria that have been advanced are that the COP should be ≤19 mm Hg (Grundmann and Heistermann, 1985) and that at least 1 L of isotonic crystal-

TABLE 6-2
Indications for Use of Parenteral Albumin[a]

Situation	Conditions
Shock	Due to blood loss
	Systolic blood pressure <80 mm Hg or central venous pressure <8 mm Hg
	Administered within 2 h after blood loss
	At least 1 L of isotonic glucose or saline solution given first
Burns	Involve >10% of body surface
	Include second- or third-degree burns
	Given >24 h after burn
Surgery	Involved retroperitoneal dissection, e.g., aortic aneurysm

[a]Adapted from Alexander *et al.* (1979).

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loiods (usually 5% glucose or 0.15 *M* NaCl) should be given first. The reviews of Boutros (1986), Erstad *et al.* (1991), and Guthrie and Hines (1991) point out that for many situations crystalloids alone are as effective as albumin, and that routine use of albumin perioperatively is not justified. The period of administration should be short, 48 h or less after surgery. For burns, albumin should not be given sooner than 24 h owing to the potential of pulmonary edema and loss through the weeping skin; a high-protein intake should be given to encourage albumin synthesis by the liver and the intravenous albumin administration then stopped as soon as possible.

Albumin therapy solely to promote wound healing appears unjustified. Administration to decrease cerebral edema is likewise not widely supported. In acute oliguria or intestinal edema causing ileus, only transient dosing was sanctioned by the 1975 Workshop. Giving albumin appears to have no value in chronic situations such as hepatic cirrhosis, nepbrosis, or protein-losing enteropathy.

Whether albumin should be given when fluid is removed to treat severe ascites (Section II,B,4a) is still debated. It does appear clear that it should not be given when less than 1.5 L of fluid is withdrawn, but that 20–60 g should be given when 10 L or so is removed in a day (Titö *et al.*, 1990).

Albumin has been injected directly into the fetus *in utero* in cases of nonimmune hydrops fetalis (see also Chapter 5, Section II,C) with good (Shimokawa *et al.*, 1988), limited (Patton *et al.*, 1986), or negative results (Lingman *et al.*, 1989). Preexisting pleural effusions or cardiac malforonations contraindicate the procedure (Maeda *et al.*, 1992).

B. Digestive Support

Giving intravenous albumin to patients who can tolerate oral food intake has been likened to pouring albumin down a drain; a good restaurant meal would be less expensive and more enjoyable. For patients receiving parenteral nutrition there is still a controversy. The controlled studies of albumin added to intravenous feedings, reviewed by Erstad *et al.* (1991), gave mixed results, one showing no significant difference and the other a reduction in hospital morbidity such as sepsis and pneumonia (Brown *et al.*, 1988). In these studies albumin was given in sufficient quantity, 25 to 37.5 g, to maintain the circulating concentration above 30 g/L.

Hardin *et al.* (1986) proceeded more aggressively to give an average of 206 g of albumin within 1–3 days to raise the albumin level in serum from 24 to 35 g/L in another group of malnourished patients receiving intravenous nutrition. The desired dose of albumin was calculated on the asumption that the volume of distribution of alhumin is 300 ml/kg body weight. The higher level of albumin was maintained without further exogenous albumin but with provision intra-

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venously of at least 90% of predicted nitrogen and caloric requirements. On the basis of studies such as these many hospitals add HSA to the intravenous formula in doses of 12.5 or 25 g/L. The considerable expense is recognized but felt to be worthwhile for the resulting lower morbidity and mortality.

Malnourished subjects with serum albumin concentration <25–30 g/L, or a COP <19 mm Hg, have been considered by some to be intolerant of full-strength oral feedings, the reason being assumed to be intestinal edema with paralytic ileus (Section II,B,4,b). Giving albumin (NSA, 25%) was judged to be helpful in restoring the tolerance to enteral nutrition in several studies (Moss, 1982; Andrassy and Durr, 1988). Patterson *et al.* (1990) more recently found that tolerance to an enteral nutrient solution was not related to the serum albumin level and that even patients with albumin <25 g/L can be fed enterally.

C. Removal of Toxins

Administering albumin to sequester fatty acids, endotoxins, and oxygen radicals and to inhibit platelet aggregation has been suggested in a stimulating article of Emerson (1989). Doing so by adding albumin in large quantity during exchange plasmapheresis to rid the body of toxins is an outgrowth of exchange blood transfusions for neonatal hyperbilirubinemia. Albumin replaces the plasma removed and is returned to the subject along with the red cells. Examples are removal of autoantibodies in myasthenia gravis, of monoclonal IgM causing circulatory difficulty due to its high viscosity, and of slowly metabolized poisons that bind to albumin.

Peritoneal dialysis with added albumin was effective in removing copper in a case of acute copper poisoning (Cole and Lirenman, 1978). An alternative approach is to enhance the effectiveness of hemodialysis in removing toxins that are tightly bound to albumin by circulating an albumin solution on the dialysate side of a high-flux dialyzer (Strange *et al.*, 1993); effective removal of bilirubin, phenol, and theophylline was claimed. An earlier technique of passing blood through columns of HSA immobilized on agarose to remove bilirubin was effective with rats but apparently has not been pursued in humans (Plotz *et al.*, 1974).

D. Imaging

Several polymerized forms of human albumin can act as agents for radiologic or ultrasonic imaging of particular areas of the circulatory system. These forms are termed microspheres, microaggregates, or microbubbles.

Aggregates less than 10 μm in diameter segregate to the reticuloendothelial system when injected into the circulation. They are prepared by polymerization

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of HSA with low concentrations of glutaraldehyde and are labeled with ^{125}I (Berghem *et al.*, 1976). Aggregates in the range 10–40 μm segregate almost completely to the lung capillaries; they are typically prepared by autoclaving the commercial 5% HSA solution at 121° for 15 min after lowering the pH to 5–6 (Robbins *et al.*, 1973). Their production and properties were reviewed by Gupta and Hung (1989a).

The chief labels used on microaggregates are short-lived isotopes such as indium-113m and technecium-93m. Because of their short half-lives, 6 h in the case of ^{99m}Tc, the isotope is generated immediately before it is coupled to the aggregated HSA. Coupling is generally performed by reduction with stannous chloride, $SnCl_2$, under nitrogen; over 90% of the ^{99m}Tc binds to the albumin. According to one manufacturer, Mallinckrodt Medical, Inc., the biological half-life of the preparation in the lung is estimated to be 10.8 h, resulting in a half-life of radiation of only 3.8 h.

Microbubbles are air-filled spheres of HSA produced by sonication at 20 kHz of heated commercial 5% albumin in a flowing stream (Hellebust *et al.*, 1993). The preparation termed Albunex (Geny *et al.*, 1993) contains about 7% by volume of these spheres, averaging 4 μm in diameter. It is useful as an innocuous target for ultrasonic imaging of large vessels and the chambers of the heart after either intravenous or intracoronary injection (Ten Cate *et al.*, 1993) or for other applications such as measuring ureteral reflux (Atala *et al.*, 1993). With Doppler scanning techniques (Hartley *et al.*, 1993) blood flow rates may be estimated.

Under the electron microscope the microbubbles are seen to be supported by a thin shell of denatured albumin about 150 Å thick. The shell is made up of several layers of HSA molecules in parallel alignment with 35-Å ridges (Christiansen *et al.*, 1994). Shell protein isolated under nondenaturing conditions shows about 60% aggregated albumin molecules; adding chaotropic agents such as GuCl lowers this to about 30%, and sulfhydryl reagents reduce it to only 5%. Thus the shell is maintained largely by hydrophobic forces but also by some intermolecular S–S bonds (Hellebust *et al.*, 1993). The oxidation is supplied by superoxide generated by the brief burst of extreme heat generated on collapse of the initially larger bubble, estimated at 170 μm in diameter, a process called cavitation by designers of propellers (Grinstaff and Suslick, 1991). Stretching the albumin layer at the vapor–liquid interface would initiate denaturation, and the heat probably causes disulfide rearrangements needed to create the sheetlike network of denatured albumin.

Native albumin, HSA or BSA, labeled with either ^{125}I or horseradish peroxidase, is commonly used as a probe of permeability of the capillaries of the pulmonary bed (see Chapter 5, Section II,A,2) and has recently been applied to intestinal and tracheal epithelia (Ma *et al.*, 1993). Detection requires autoradiography or immunohistochemical techniques, preferably at the ultrastructural level.

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E. Drug Delivery

Microspheres have other uses in sustaining the release of therapeutic drugs injected into the circulation (Morimoto and Fujimoto, 1985; Gupta and Hung, 1989b). The drugs are entrapped in the aggregated albumin as it is prepared, either by heating or exposure to glutaraldehyde. Concentrations of albumin as high as 50% (w/v) appear to yield better cross-linking (Rubino *et al.*, 1993). A second coupling agent, formaldehyde or carbodiimide (Sheu *et al.*, 1991), is sometimes employed as well to bind the drug.

Many of the drugs delivered in this manner are antitumor agents: Adriamycin (Gupta *et al.*, 1990), doxorubicin (Cummings *et al.*, 1991), deoxyfluorouridine (Sheu *et al.*, 1991), cisplatinum (Nishioka *et al.*, 1989), and cyclophosphamide (Vural *et al.*, 1990). Their release from the microspheres is prolonged, and they tend to lodge in the reticuloendothelial system and track the spread of tumors. Virus particles copolymerized into albumin microbeads may serve as vaccines; they show delayed release on injection into rabbits, and generate humoral antibodies as effectively as do virus particles in Freund's incomplete antigen (Newman, 1990). Microspheres incorporating the antibiotic, chlorhexidine, appear to be useful in preventing urinary tract infections when coated on urinary catheters (Egbaria and Friedman, 1990).

More specific localization is obtained with magnetic microspheres, prepared by including the mineral magnetite during their preparation. These can be drawn to a desired area by a powerful external magnet (Pande *et al.*, 1991). Delivery of *cis*-hydroxyproline to scarred areas in such magnetic microspheres reduces the content of collagen in the scar and inhibits its growth; to date results have only been reported with a rat-tail model (Iannotti *et al.*, 1991).

Monomeric albumin as well as aggregates can be used in drug delivery. Just as albumin aids in the removal of tightly bound compounds such as bilirubin, it can serve to deliver heme in the treatment of acute intermittent porphyria (Bonkovsky *et al.*, 1991). The sustained release of heme mollifies the overproduction of porphyrin, which is characteristic in such cases. In treating malignant melanoma through infusion of IL-2 plus subcutaneous injection of α-interferon, addition of albumin to the infusion decreased the toxicity and appeared to aid the response (Cassidy *et al.*, 1991). The mechanism of this beneficial effect is not known; albumin is known to bind interferon (Chapter 3, Section I.E.5) but not interleukins.

Tumor therapy through delivery of radioiodine compounds coupled to albumin (Sinn *et al.*, 1990) or of methotrexate coupled to albumin and then to a surface antigen of mammary tumor cells (Eodo *et al.*, 1987) is under investigation. Killing of *Mycobacterium tuberculosis* harbored by mouse macrophages was achieved by coupling the drug, *p*-aminosalicylate, to maleylated BSA, which is readily assimilated by macrophages (Chapter 5, Section III.C.1) (Majumdar and Basu, 1991).

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The free-radical-destroying enzyme, superoxide dismutase, has been administered following myocardial injury to lessen toxic effects of oxygen radicals, but its short survival time in the circulation lessened its efficacy. Coupling to albumin improved the stability and circulation time of this agent over those of the free enzyme (Mao and Poznansky, 1989).

F. Coating Surfaces

Makers of *in vivo* devices constantly seek better means of preventing the buildup of clots on surfaces of blood vessel grafts and vascular catheters. A thin coating of albumin appears to passivate these surfaces, minimizing adhesion and aggregation of platelets with subsequent thrombus formation (Kottke-Marchant *et al.*, 1989). Simple preflushing of an IBM red cell separator with 4% HSA, for instance, reduced the incidence of clots sixfold and platelet deposition by 95% (Tsai *et al.*, 1990).

In coating of knitted polyester grafts, albumin cross-linked with glutaraldehyde or carbodiimide is useful; it both decreases leakage and limits the adherence of leukocytes as well as platelets (Kottke-Marchant *et al.*, 1989). Such coatings can be sterilized with γ-radiation, either dry or in saline, but become stiff on autoclaving (Baquey *et al.*, 1987).

Efforts to improve the attachment of HSA to surfaces of these devices continue. Most of these involve provision of chemical groups favoring attachment on the surface, such as carboxyls of acrylic acid, to which proteins can be coupled with carbodiimide (Kang *et al.*, 1993). More specific for albumin are LCFAs bonded to silicone rubber (Tsai *et al.*, 1990) or polyurethane (Pitt et al., 1988), or Cibacron Blue dye covalently bound to polyetherurethane by an arm of dextran bonded via an acrylamide link (Keogh and Eaton, 1994). Here the idea is that the selective and reversible binding of native albumin to these ligands will provide a constantly renewed biocompatible coating. The Cibacron Blue product bound 0.3 μg HSA/cm^2, double the usual amount bound to surfaces (Chapter 2, Section II,C,2,d).

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7

Practical Aspects: Albumin in the Laboratory

This final chapter considers the preparation and use of albumin in the laboratory, with the emphasis on practice rather than theory. Starting from a list of properties of albumin that are useful in its isolation, it will review the ways in which these have been applied to isolate albumin both in the laboratory and on the commercial scale. Then it will examine numerous low-level impurities that can adulterate even the cleanest albumin preparations, and present some of the many applications of albumin in life science research. In the final section I have set forth a sort of cookbook for colleagues interested in using albumin in their research, recipes that my close associates and I have found effective in isolating and manipulating albumin in the laboratory.

I. METHODS OF PREPARATION

The isolation of serum albumin is invariably based on one or more of the properties listed in Table 7-1. Basically these are its solubility, which is often the highest of any protein present, its negative charge at pH >5, its affinity for hydrophobic substances, and its unusual stability. Except in the presence of high salt concentrations, e.g., 2 *M* ammonium sulfate, its solubility generally rises with temperature in the –20° to +40° range. [For reviews of albumin preparation see Watson (1965), Rothstein *et al.* (1977), and More and Harvey (1991).]

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TABLE 7-1
Properties Useful in Isolation of Albumin

Property	Comments	
Solubility	High	Low
	Distilled water (>500 g/L)	Minimum near pH 5
	0.50 saturation (2.05 *M*) ammonium sulfate, 25°, pH 6.5 (>15 g/L)	As for high solubility, pH ≈4.5; 0.75 saturation (3.1 *M*) ammonium sulfate, 25°
	40% (v/v) ethanol, pH 5.8, μ = 0.005, –5° (5 g/L)	As for high solubility, pH 4.9, (0.03 g/L)
Charge	Usually most cathodic band on electrophoresis or isoelectric focusing	
	Usually first protein eluted from cation-exchange medium	
	Usually last protein eluting from anion-exchange medium	
Affinity	Retained by Cibacron Blue, μ < 1 *M*	
	Immobilized LCFAs, hydrophobic media at neutral pH	
	Immobilized antialbumin, pH 4–8	
	Immobilized Cu^{2+}	
Stability	Soluble (≈ 10 g/L) in 1% trichloroacetic or perchloric acid with 80–95% ethanol or acetone	
	Not denatured by 60°, 10 h, 50 g/L albumin, pH 6.8, with 4 m*M* caprylate	

A. Laboratory Purification

1. Solubility

The high solubility of albumin (Chapter 2, Section II,B,3) makes this principle attractive as a first step in its isolation. Exhaustive dialysis against distilled water will often remove many accompanying proteins and leave the albumin in solution. Addition of ammonium sulfate to half-saturation (2.05 *M* at 20–25°, Table 7-1) precipitates all but transferrin and traces of other serum proteins (Fig. 7-1, lane b). In the cold alcohol procedures, albumin is essentially the only plasma protein remaining in solution at 40% ethanol (Table 7-1). King (1972) has proposed an ammonium sulfate gradient procedure for purification.

In recovering the albumin from solution several techniques make use of fractional precipitation as a purification step. The solubility of albumin, like that of most proteins, is minimal at its isoelectric point; in the case of albumin, however, with its high charge, this effect is much stronger, and its solubility increases manyfold just a pH unit away from its isoelectric point near pH 5 (Hughes, 1954). Almost total precipitation of albumin occurs when the pH of a

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Fig. 7-1. Polyacrylamide gel electrophoresis of human albumin at various stages of purification. (a) Whole serum; (b) 2.1 M ammonium sulfate supernatant; (c) precipitate from fraction at pH 4.4; (d) plasma protein fraction (PPF) (Cutter Laboratories); (e) Fraction V from cold-alcohol method (Bayer; formerly Miles, inc.); (f) albumin prepared by the TCA–alcohol method; (g) crystalline albumin (Bayer; formerly Miles, Inc.); (h) monomeric mercaptalbumin prepared by chromatography (see text). T, Transferrin; D, albumin dimer. Reproduced from Peters (1975) by permission of Academic Press.

2.05 *M* ammonium sulfate solution is lowered from pH 6.5 to pH 4.5 (Table 7-1; Fig. 7-1, lane c). At 40% ethanol (Table 7-1) the solubility drops over 100-fold from pH 5.8 to pH 4.9.

Addition of water-soluble polymers of high molecular weight can also effect fractional precipitation. The mechanism has not been defined. Polyethylene glycol (PEG) of molecular mass ≈6000 Da has been most frequently employed; at pH 7.0 albumin is the last of the plasma proteins to precipitate as the PEG concentration is raised, coming out of solution at 14–20% (w/v) of the polymer (Polson *et al.*, 1964). Indeed, PEG is the basis for a simple and rapid isolation procedure for albumin (Gambal, 1971). The need for removal of the polymer from the final suspension is a drawback, and requires that the PEG be precipitated with alcohol or the albumin adsorbed on an ion-exchange column (Vasileva *et al.*, 1981).

Large organic cations will form insoluble complexes with negatively charged proteins. The acridine, Rivanol, was first reported as a selective precipitant by Hořejší and Smetana in 1954 and has been the most widely used. Kaldor *et al.* (1961) have studied the interaction; they found that ≥7 mol acridine/mol albumin will precipitate albumin at alkaline pH, and showed the expected solubilizing effect of added salts on complex formation. At pH 8 albumin and the more acidic plasma proteins, largely α-globulins, are precipitated by 0.84% Rivanol. Further steps are needed to purify the albumin from the precipitate.

Cationic metals, e.g., Zn^{2+} or Ba^{2+}, by the same principle will aid in precipitating negatively charged proteins. They have been employed to minimize the concentration of ethanol needed in later versions of the Cohn procedure (Surgenor *et al.*, 1960). The action of bound zinc is to shift the effective isoelectric point of albumin from 4.6 to 7 and decrease its solubility in the neutral range.

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Strong acids, particularly those with large anionic groups, together with organic solvents will precipitate most plasma proteins and leave albumin in solution. This phenomenon dismayed this unwitting author in causing a carefully isolated albumin preparation to disappear on washing with ethanol to remove lipids after TCA precipitation. Michael (1962) has studied the action of different acids and different solvents; hydrochloric, formic, and trichloroacetic acids were about equally effective, and 70–95% methanol, ethanol, or acetone could serve equally well. Hydrochloric acid or TCA together with ethanol has been used for clinical determinations of albumins and globulins in serum (Chapter 6, Section I.A). The albumin can be precipitated with diethyl ether or by addition of sodium acetate to pH ≈5, and is seen as a pure protein with a small amount of dimer (Fig. 7-1, lane f). Iwata *et al.* (1968) have presented details and critically reviewed earlier publications on this technique. The same principle is useful in recovering albumin from its precipitate with antibody (Kallee *et al.*, 1957; Peters, 1958).

Crystallization is the classical method of purification of chemical compounds, and has been widely employed with albumin. Commercial crystalline albumin appears electrophoretically pure except for considerable (≈5%) dimer and traces of higher oligomers (Fig. 7-1, lane g). Crystallization was discussed in Chapter 2 (Section II,A,3); it is seldom used as a purification step in the laboratory.

2. *Charge*

The high negative charge of albumin is an important factor in methods for its isolation. Preparative electrophoresis, either by continuous flow or by zone electrophoresis in a supporting bed, is applicable to isolations at the 200- to 400-mg level. For the latter, Sephadex has replaced starch as the solid medium. A 1 × 19 × 35-cm bed of Sephadex G-25, for instance, can fractionate 10 ml of serum overnight (Peters and Hawn, 1967). An advantage of the flat bed is that the separation can be monitored at any time by taking a "print"—touching the edge of a narrow strip of filter paper to the bed, which wets by capillary action—then fixing and staining for protein. Isoelectric focusing and chromatofocusing techniques have high resolution but are generally applied for analytical rather than preparative purposes.

Ion-exchange chromatography is the workhorse of albumin purification in the laboratory. The earliest media used were silica gel, diatomaceous earth, and hydroxyapatite. Since its introduction by Sober *et al.* (1956), the anion exchanger, DEAE, fixed to cellulose, Sephadex, or Sepharose, has been popular (Ramsden and Louis, 1973). With a salt gradient at pH 7–8 albumin is the last of the plasma proteins to be eluted.

Cation exchangers have the advantage that albumin is the first major protein to be eluted (Table 7-1) and so is more likely to emerge in a sharp peak. Carboxymethyl-Sepharose, sulfoethyl-Sephadex (Hagenmaier and Foster, 1971),

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and *N*-methylpyridinium polymer (Nishimura *et al.*, 1990) are examples. Many of the ion-exchange media are available commercially for use in the rapid and convenient HPLC technique.

3. Affinity Chromatography

In this technique a ligand is fixed covalently to a solid support and albumin is allowed to bind to it, then is eluted by a change of conditions after undesired proteins are washed away. The most-favored ligand for albumin is the triazine dye, Cibacron Blue F3-GA, linked to Sepharose as introduced by Travis *et al.* (1976). At low salt concentrations and neutral pH albumin is 98% bound, and globulins can be largely washed off with 1 *M* NaCl (Gianazza and Arnaud, 1982). The albumin is so tightly bound that its elution requires 2 *M* NaCl or a chaotropic agent such as 0.5 *M* NaSCN.

The dye is a general ligand for affinity chromatography of enzymes that bind to substrates having the "dinucleotide fold" configuration; it has been suggested that it binds to the bilirubin site on HSA (Site I). Presaturation of HSA with bilirubin did not affect the binding, however, whereas prior addition of fatty acids $>C_{12}$ reduced it, suggesting that it occupies LCFA binding sites (Metcalf *et al.*, 1981). Cibacron Blue is useful chiefly with preparation of human albumin; most other species bind the dye less strongly (Mahany *et al.*, 1981). It cannot be the basis for preparation of pure albumin in a single step, but is useful for selective removal of albumin from plasma (Travis and Pannell, 1973). Gianazza and Arnaud (1982) have made a careful study of the elution profiles at pH 5, 7, and 9. Commercial batches of the dye should be evaluated prior to use (Hanggi and Carr, 1985).

Other hydrophobic ligands have been applied to the isolation of albumin. Among these are 4-phenylbutylamine (Hofstee, 1973) and bromosulfophthalein–glutathione attached to Sepharose 4-B. The latter system had been used for the isolation of glutathione *S*-transferase (Clark and Wong, 1979). A dichlorotriazine dye, CI reactive Blue 4, has been applied in a fluidized bed of perfluorocarbon emulsion as a semicontinuous procedure for albumin isolation from plasma (McCreath *et al.*, 1992); albumin purity was from 85 to 95%. Bilirubin (Hierowski and Brodersen, 1974) and palmitate are highly selective ligands; desorption from immobilized bilirubin can be effected by competing ligands such as salicylate or sulfa compounds, but detergents (Wichman and Andersson, 1974) or strong alkali and alcohol (Peters *et al.*, 1973) are required to remove albumin from palmitate.

4. Size: Stokes Radius

Neither ultracentrifugation nor differential ultrafiltration has become a useful technique in isolation of albumin owing to technical difficulties. In therapeutic

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plasmapheresis to remove immunoglobulins, however, differential membrane ultrafiltration is able to recover and return to the body 80% of the albumin in the plasma removed (Ding *et al.*, 1991).

The permeation/exclusion or gel filtration procedure is widely used to remove larger proteins in conjunction with other principles such as ion exchange. Passage through a column of Sephadex G-100 in a neutral buffer, for example, is an effective means of isolating albumin monomer, and is frequently applied as the last step in a preparative scheme (Fig. 7-1, lane h). Here the albumin molecules enter the weakly cross-linked dextran beads and are eluted only after nearly a whole column-volume equivalent of buffer has been applied. Isolation of mercaptalbumin (Section II,B,2) is another method of obtaining albumin monomer.

5. *Miscellaneous Isolation Methods*

Porath and co-workers at Uppsala have developed a series of immobilized metal ion columns for chromatography of albumins (L. Andersson *et al.*, 1991). Of 10 albumins studied all were retained on Cu(II) columns; all but dog albumin were retained on Ni(II) columns. Because chicken, pig, and dog albumins lack the specific X-X-L-His amino-terminal site for Cu(II) binding (Chapter 3, Section II,A,1; Fig. 4-3), binding at this site apparently does not predominate in this interaction. The technique does not appear to have been developed to the point of isolation of albumin from plasma.

Albumin can be isolated in small quantities by generic immunoaffinity techniques. Here antialbumin antibodies are covalently linked to a support such as Sepharose and the albumin is allowed to bind, then is eluted at pH <3 or >9. (Capacity in my experience is ≈1 mg rat albumin per milliliter of anti-RSA-agarose; details of the preparation and elution conditions are given in Peters and Reed (1980).)

A technique not for isolation of albumin but for removal of unwanted glycoproteins is affinity chromatography with lectins such as concanavalin A (ConA) (Ikehara *et al.*, 1977). ConA binds to mannosyl groups, so will remove most cell-derived glycoproteins. It would not be expected to remove the ≈1% of HSA molecules containing glucosyl groups from nonenzymatic glycosylation; these can be removed on boronic acid columns (Chapter 6, Section II,B,3,a).

6. *Complete Schemes for Laboratory Purification*

Numerous schemes for albumin purification on a laboratory scale have appeared. Feldhoff *et al.* (1985) used carboxymethyl- and DEAE-cellulose chromatography sequentially on a 2.05–3.1 *M* ammonium sulfate fraction, then purified further on Cibacron Blue F3GA-agarose. Although their procedure was

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written for use with mouse ascites fluid, it should be equally applicable to plasma. The product was described as homogeneous on polyacrylamide gel electrophoresis.

Earlier, Curling *et al.* (1977) at Pharmacia Fine Chemicals, Uppsala, described a scheme for preparation of HSA of over 95% purity from plasma in 95% yield. They removed the cryoprecipitate (the precipitate obtained on thawing) in 12% PEG at pH 8, then precipitated crude albumin with 25% PEG at pH 4.6. The albumin fraction was purified first on DEAE-Sephadex or DEAE-Sepharose CL-6B, next on sulfopropyl-Sephadex C-50, and then desalted on Sephadex G-25. By use of suitably large equipment 50–150 L of plasma per week could be treated.

A recent, smaller scale application of ion-exchange chromatography is that of Nochumson (1992) using microporous plastic silica sheets formed as "ACTI MOD" cartridges. At pH 7.2 and 0.1 *M* NaCl the underivatized medium absorbs serum proteins more basic than albumin. The crude albumin is then absorbed on a quarternary amine-silica cartridge by anion exchange at the same pH and ionic strength, and eluted with 0.25 *M* NaCl. The albumin product was described as >95% pure.

For investigators equipped to handle cold ethanol techniques, Hao (1979) has published a simple two-step procedure. Human plasma, diluted to contain 1.2% protein and 42% ethanol at pH 5.8, $\mu = 0.09$, -5°, is centrifuged or filtered with filter aid at that temperature. The filtrate or supernate is adjusted to pH 4.8 with 2 *M* sodium acetate buffer, which brings maximal precipitation of albumin. The albumin is separated and the cake is suspended in water and the remaining ethanol is removed by dialysis or gel filtration. In my hands this procedure yielded albumin of ≥98% purity but only in about 40% yield from 3-mL samples of serum; the more experienced Hao obtained 93% yield and >99% purity. A subsequent version (Hao, 1985) adapts this procedure to the pilot-plant scale (≈30 L).

For the preparation of mercaptalbumin (HMA), precipitation as the HSA mercury dimer (Hughes and Dintzis, 1964) offers a straightforward method (Chapter 2, Section II,B,5). Dialysis against 1 m*M* cysteine suffices to remove the mercury. Immobilized disulfide compounds on silica have been introduced more recently for preparing HMA (Millot and Sebille, 1987). Ion-exchange chromatography can also separate HMA from nonmercaptalbumin (Era *et al.*, 1988) (discussed further in Section III).

B. Commercial Purification

As noted in Chapter 6, the annual production of purified HSA exceeds 300,000 kg. This is isolated from plasma of donors screened for hepatitis B virus and human immunodeficiency virus; the plasma is now frequently obtained by

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plasmapheresis, which greatly increases the yield from a single donor. The human placenta is also a useful source, particularly in France.

HSA Fraction V, >96% albumin, is the major product obtained today from human plasma. The American Red Cross ships its plasma in the frozen state, and Factor VIII (antihemophilic factor concentrate) is prepared from the cryoprecipitate obtained on thawing. Other useful derivatives are clotting Factors IX and I (fibrinogen) and immune globulin. Plasma protein fraction (PPF), >83% albumin (Finlayson, 1980), is a more crude plasma substitute (Fig. 7-1, lane d) that can be prepared by a continuous small-volume mixing technique (Cash, 1980).

For details and more background information on commercial plasma fractionation the reader is referred particularly to the chapter by More and Harvey (1991), as well as to the brief review by Vandersande (1991), the earlier reviews by Rothstein *et al.* (1977) and Finlayson (1980), and the comprehensive volume by Schultze and Heremans (1966). The description by the legendary E.J. Cohn of the wartime plasma fractionation program (Cohn, 1948) and the official U.S. Army account of the same era (Coates and McFetridge, 1964) are interesting reading for the historical aspects. Figure 7-2 shows equipment in a modern commercial fractionation plant.

1. Alcohol Fractionation Procedures

The key method resulting from the program at the Harvard Physical Chemistry Laboratory, known as Cohn Method 6, has been the mainstay of commercial fractionation since the 1940s. From it the term Fraction V for nearly pure HSA has arisen, because albumin is the precipitate of the fifth step of the original procedure. As listed in Table 7-2, citrated plasma is diluted to 5 g/L protein and alcohol added to 8% at –3° for the removal of Fraction I, largely fibrinogen. Increase of ethanol to 25% at –5° yields combined Fractions II and III, with most of the γ-globulins. Diluting the ethanol to 18% while lowering the pH to 5.2 yields Fraction IV-1, chiefly α-globulins, including ceruloplasmin, and 40% ethanol at pH 5.8 precipitates Fraction IV-4, containing transferrin along with both α- and β-globulins.

The key step in albumin isolation (Tables 7-1 and 7-2) is the lowering of pH to 4.8 at 40% ethanol. The precipitate is Fraction V, >96% albumin (Fig. 7-1, lane c), in a yield of 83% of the albumin of the plasma or 92% of the total albumin found in all of the fractions.

Kistler and Nitschmann in 1962 introduced an abbreviated procedure for removal of the globulins. It consisted of combining the second and third steps of Cohn Method 6 (Table 7-2) by going directly to pH 5.85 and 19% ethanol after Step 1. Yield was reported as 99% of albumin of 99% purity. Because there is little demand for the intermediate fractions this simpler process has

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Fig. 7-2. A modern commercial plant for the manufacture of bovine serum albumin. Courtesy of Bayer (formerly Miles Laboratories, Inc.).

TABLE 7-2
Cohn Method 6 for Human Albumin Isolation[a]

Conditions						
[Ethanol] % (v/v)	pH	Ionic strength	Temperature (°C)	[Protein] (g/L)	Material	Yield (g/L albumin)
—	—	—	—	—	Plasma	36.3
8	7.2	0.14	−3°	5.1	Fraction I	0.2
25	6.9	0.09	−5°	3.0	Fractions II + III	0.8
18	5.2	0.09	−5°	1.6	Fraction IV-1	0
40	5.8	0.09	−5°	1.0	Fraction IV-4	0.9
40	4.8	0.11	−5°	0.8	Fraction V	29.9
—	—	—	—	—	Supernate V	0.8
					Total	32.6

[a]From Cohn *et al.* (1946).

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attained wide application. The two-step method of Hao described in the preceding section as in the pilot-plant stage combines the first four precipitations into a single step.

Heat-shock processes, so named for their denaturation of most globulins at temperatures above the pasteurization range, vary from mere heating at 60–75° in the presence of caprylate to the technology of Schneider *et al.* (1975), which has been adopted by the German Red Cross and fractionation centers in a few other countries (More and Harvey, 1991). In this procedure diluted plasma is heated at 68° in 9% ethanol at pH 6.5 with added caprylate. In the final stage of the process albumin is precipitated with PEG. A later version (Hansen and Ezban, 1980) combines PEG separation and heat treatment; nonalbumin proteins are denatured at 60° at pH 4.6. Albumin of ≈100% purity is recovered in 90% yield, and the shortened processing time and lack of cooling equipment make the heat-shock method popular for its low capital costs. In the United States heat shock is in vogue for bovine albumin isolation but is not currently authorized for preparing HSA for *in vivo* use.

2. *Chromatographic Methods*

Ion-exchange chromatography can produce albumin of high purity using mild conditions. Advantages of the chromatographic procedure are absence of solid–liquid separation steps and of need for refrigeration, plus the ability to recover both albumin and other plasma products in high purity and using mild conditions. Disadvantages are seen, however, in the special concern needed to maintain sterility and prevent contamination of the solid media, and to guard against ligand leakage from the bed into the product.

The lower capital equipment costs cause chromatography to be applied mainly in small-scale commercial installations. More and Harvey (1991) list 17 process options that have been published; a prime example is the facility at the Winnipeg Rh Institute (Friesen, 1987). Nearly all of the schemes utilize an anion exchanger based on DEAE, usually at pH 8, after initial filtration or gel filtration of plasma, then apply the desorbed albumin fractions to a carboxymethyl or sulfopropyl cation exchanger near pH 5. Batch sizes can be as high as 800 L of plasma. Yields have been reported to be 80–85% of plasma albumin with >98% purity and <2% polymer.

3. *Miscellaneous Procedures*

Precipitation of albumin with Rivanol at pH 8 (see above) has been used by Behringwerke with subsequent purification by ammonium sulfate fractionation (More and Harvey, 1991). The Rivanol is dissociated at pH 5 with 5% NaCl and removed by adsorption on charcoal. The Institute Merieux isolated albumin from

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placental extracts using ethanol-chloroform treatment followed by TCA–ethanol fractionation, precipitating the albumin by neutralization (Rothstein *et al.*, 1977).

4. Final Treatment and Pasteurization

Precipitated albumin Fraction V, usually stored as a frozen cake, is thawed and brought to ≈10% ethanol. The ethanol was originally removed by lyophilization, and later by dialysis or gel filtration, but "diafiltration"—ultrafiltration with addition of a washing solution—is now more convenient and permits facile adjustment of protein concentration, pH, and ionic strength.

In the commercial process sodium caprylate and/or sodium acetyl-L-tryptophanate are added just before the solution is bottled in individual containers, usually at 5% (50 g/L), 20%, or 25% concentration. By FDA rules HSA is then heated for 10–11 h at 60 ± 0.5° in the final container. Further observation for development of turbidity during 14 days at 30–32° screens for bacterial contamination.

The tale of how *N*-acetyltrytophan came to be a standard method of protecting commercial albumin preparations during pasteurization has been recalled by John T. Edsall (1984). A virus-safe product was desperately needed during World War II. Both chloride and acetate had been found by J. Murray Luck and associates to be somewhat protective of albumin during heating; Edsall suggested trying propionate, and this led to learning that caprylate was some 20 times as effective (Ballou *et al.*, 1944) as the best agent to date, phenylacetate, allowing heating to 82° before flocculation occurred. Acetylphenylalanine was suggested by Hans T. Clarke, and acetyltryptophan by Laurence E. Strong, at the Harvard Pilot Plant. Tryptophan was suggested because it is the amino acid in which albumin is most deficient, and so might have nutritional benefit! The stabilizing effect of 0.04 *M* acetyltryptophan proved to be so great that the albumin in 25% solution could withstand the heating in its final container at 60° for 10 h. The effectiveness of acetyltryptophan and octanoate appears to reflect a strong conformational change originating at Site II that makes the albumin molecule more resistant to the denaturing effect of heating. A single LCFA, bound nearby in domain III, has a similar effect.

FDA specifications for the final product, HSA Fraction V, USP, are 5 ± 0.3% (50 g/L) albumin (in the 5% preparation), >96% albumin, pH 6.9 ± 0.5, and $A_{403\ nm} < 0.25$, the latter being a test for hematin or hemoglobin (U.S. Congressional Register, 1987, 640-81e,f). Further requirements are potassium <50 μmol/g protein, sodium 130–160 m*M*, and stability in storage for 5 years under refrigeration or 3 years at 37° (Hink *et al.*, 1970); no turbidity must appear after 50 h at 57° (see also Chapter 6, Section III).

Concern over possible viral contamination of HSA prepared in this manner has been alleviated by (1) screening of all donors for antiviral antibodies; (2) demonstration that added viruses tend to partition with fractions precipitating

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earlier than albumin and to be inactivated by ethanol (Morgenthaler and Omar, 1993); (3) demonstration of the high efficacy of pasteurization conditions in killing added viruses, particularly HIV, which is destroyed by $>10^9$ in 4 min at 60° (Wah *et al.*, 1986); and (4) an unblemished record of clinical safety from virus transmission during 45 years of use (Horowitz, 1990; Cuthbertson *et al.*, 1987). Placenta-derived albumin appears to have an equally good record (Pla *et al.*, 1974; Rothstein *et al.*, 1977; Grandgeorge and Veron, 1993).

C. Recombinant Production

Interest in recombinant production of HSA for parenteral use is rising as the progress of fermentation technology makes large-scale manufacturing more feasible. Driven partly from the desire to avoid any possibility of viral contamination, however slight, from the donor-derived albumin, and by the increasing worldwide demand for albumin, several large firms are actively pursuing recombinant synthesis of HSA. A prediction of human albumin costing $1/g may be a reality in the next decade. Surely major changes in the plasma fractionation field will follow. [For a recent review see Storch (1993).]

Numerous host organisms have been tested, with yeasts being the most successful. Following his cloning of cDNA for HSA in *Escherichia coli*, Lawn (1983) applied for a European patent for the process. The same year Scandella and McKenney (1983) sought a United States patent for cloning the HSA gene in the same organism. In these procedures and in early trials with yeast, *Saccharomyces cerevisiae* (Latta *et al.*, 1987; Quirk *et al.*, 1989), the albumin was not secreted but had to be extracted under denaturing conditions (8 *M* urea, pH 10, with 2-mercaptoethanol), then caused to refold into a hoped-for native state. Expression in transformed plants (Sijmons *et al.*, 1990) carried the same burden. Attempts to carry out signal peptide cleavage and secretion in *Bacillus subtilis* (Saunders *et al.*, 1987) gave only low yields.

In 1986 Delta Biotechnologies, Ltd., of Nottingham filed a European patent application for expression and accompanying cleavage and secretion from cultured yeast, *Saccharomyces cerevisiae* (Hinchcliffe and Kenney, 1986). This firm, allied with the large brewing company, BASF, has the potential for high-capacity production and has long experience in fermentation of the organism. As of this writing the Green Cross Corporation of Japan (Okabayashi *et al.*, 1991) and Vepex-Biotechnika, Ltd., of Hungary (Kalmän *et al.*, 1990) are also pursuing secretion of recombinant HSA from *S. cerevisiae*, and Rhône-Poulenc Rorer in France is testing *Kluyveromyces* yeasts (Fleer *et al.*, 1991). Other companies are no doubt pursuing recombinant albumin production but have not published results as yet.

Typically the cDNA of human liver mature albumin mRNA is fused into an appropriate plasmid, which is then used to transform yeast cells by standard

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molecular biological techniques. Various leader sequences have been inserted and tested for optimal cleavage. Codon usage of the added leader is designed to be optimal for translation in yeast. Sleep *et al.* (1991) capitalized on the known ability of the yeast KEX2 protease to cleave human proalbumin at its Arg-Arg site (Chapter 5, Section I,D,4) and the intrinsic yeast protein MFα-1, at its Lys-Arg site; their selected leader sequences ended with Lys-Arg or Arg-Arg. Yields of mature albumin secreted into the culture medium were 45 to 55 mg/L. About 3% of a 45-kDa HSA fragment, residues 1 to ≈409, was usually found as well; its origin was not apparent, but neither removal of a potential KEX2 Lys-Lys site at residues 413–414 nor optimization of the codon usage for yeasts in that region to prevent "ribosomal stalling" precluded appearance of this 45-kDa fragment. With the native HSA prepro leader, small amounts of a >66-kDa HSA representing pro- or prepro-HSA also appeared in the culture medium.

The French firm employed the native HSA prepro leader in *Kluyveromyces lactis* (Fleer *et al.* 1991). The KEX1 convertase of this industrial strain of yeast appeared to function well based on a report of secretion of HSA at "several grams per liter."

Okabayashi *et al.* (1991) also tested another approach, that of utilizing the intrinsic yeast signal peptidase and skipping the propeptide insertion. Their clones included the native human albumin presequence, a human albumin presequence with the cleavage site modified for more optimal signal peptidase action (Chapter 5, Section I,C,2,a), and the yeast invertase presequence. Higher yields of secreted mature HSA, 30 to 85 mg/L, were found with the presequence leaders, compared to 31 mg/L with a leader ending in a propeptide.

The group in Hungary *synthesized* the gene coding for mature HSA, the longest synthetic gene thus far described (Kalmän *et al.*, 1990). Construction of the 1761-bp DNA used 24 69- to 85-bp oligonucleotides, which were first linked to form four larger fragments. An accompanying advantage is the opportunity to select codons for optimal translation of the entire chain by yeast. With the signal peptide sequence of a yeast acid phosphatase as leader, secretion of HSA by *S. cerevisiae* was about 10 mg/L.

The secreted albumin, as isolated by ultrafiltration, elution from Cibacron Blue columns with 2 *M* sodium thiocyanate, and gel filtration (Okabayashi *et al.*, 1991), generally appears to be native, as judged by amino- and carboxy-terminal amino acid sequences, immune reactivity, and binding of chiral drugs (Fitos *et al.*, 1993). Clinical trials were said to have begun in 1992 with one preparation (Storch, 1993), which would imply production at least on a pilot-plant scale.

Problems must lie ahead, however, with usage in humans. Traces of yeast proteins could be a cause of anaphylaxis. Sequence errors can appear in the HSA cDNA, and may be a source of trouble. The synthetic genes in particular are prone to incorporate sequence errors; only 45% of synthetic clones were error free (Kalmän *et al.*, 1990). In good laboratory practice the whole sequence is

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verified with each batch of HSA produced even with natural HSA cDNA. Considering these and unforeseen pitfalls, plus the long and careful assessment period by the FDA, recombinant albumin as a clinical therapeutic agent would seem to be a few years in the future.

II. FURTHER PURIFICATION; ALBUMIN HETEROGENEITY

Like those of any natural product, preparations of albumin contain traces of other materials as well. Although these impurities appear harmless when administered to humans intravenously, they may be detrimental to the action of *in vitro* systems, and the investigator using albumin in the laboratory should be aware of the possibility of their presence and of their lot-to-lot variability.

A. Associated Substances

1. Low Molecular Weight Substances

Table 7-3 lists some of the small-molecule materials identified in crude HSA Fraction V powder. The major substance is water; lyophilized proteins generally contain 3–5% residual water, which in the author's experience may rise to 15% if a container is opened frequently in humid surroundings. Inorganic materials, collectively seen as 1–3% ash, include chloride and heavy metals, particularly iron, zinc, copper, and aluminum. The caprylate and acetyl-L-tryptophanate levels in Table 7-3 are those prescribed by the FDA to be added prior to pasteurization, 0.08 mmol each per g albumin (5.4 M/M). The listed amounts were found in Fraction V solutions, but are not readily dialyzable; the indole group can interfere with spectral measurements in the near-ultraviolet (Bargren and Routh, 1974). Total fatty acid levels (MCFAs plus LFCAs) of 3–9 M/M albumin have been reported for Fraction V powders (Birkett *et al.*, 1978; Imada *et al.*, 1981). Small amounts of other lipids occur, namely, 0.08 M/M phospholipids and <0.015 M/M cholesterol (Rosseneu-Motreff *et al.*, 1970). Citrate is another major organic impurity, a residue of the sodium citrate anticoagulant used in plasma collection, even with plasmapheresis.

Many of the metallic constituents are probably acquired during processing, and improved technology including diafiltration has lowered their levels since 1967, when the analyses were made. Copper and nickel, although tightly bound by HSA, are found at several times the molar ratios occurring in plasma, 0.004 and 0.00001 (Leach and Sunderman, 1985), respectively. Aluminum originates from the diatomaceous earth used as a filter aid; it is a source of concern in patients receiving large amounts of albumin for treatment of burns (Klein *et al.*, 1990) and particularly for patients on hemodialysis therapy. European manufacturing pro-

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TABLE 7-3
Low Molecular Weight Substances Detected in Crude HSA Fraction V Powders

Substance	Concentration[a] (μg/g)	M/M	Reference[b]
Water	30–50 mg/g	- -	1
Ash (sulfated)	10–30 mg/g	—	1
Cl, as NaCl	1–13 mg/g	1–16	1
Aluminum	1–27	0–0.07	2
Antimony	0.01–0.08	<0.0001	3
Calcium	120	0.21	4
Chromium	0.2–0.7	0–0.001	3
Cobalt	0.04–0.17	0.0002	3
Copper	5–32	0.01–0.04	3
Iron	24–98	0.03–0.12	3
Lead	<5	<0.002	1
Magnesium	21	0.06	4
Manganese	0.2–0.4	<0.0005	3
Mercury	0.1–0.3	<0.0001	3
Nickel	0–1.5	0.002	5
Scandium	0.0002	<0.0001	3
Silver	0–0.1	0.0001	3
Zinc	12–152	0.01–0.16	3
Acetyltryptophanate	21.5	5.4	6
Caprylate	11.5	5.4	6
Carbohydrate, as hexose	<1	<0.4	7
Citrate	9.0 mg/g	3.4	8
Hematin	<0.18 mg/g	<0.02	9

[a]Concentrations given in μg/g except where noted otherwise.

[b]Key to references: (1) Bayer (formerly Miles Laboratories) (1992), (2) Klein *et al.* (1990), (3) Malvano *et al.* (1967), (4) Armour CRG-7, (5) Leach and Sunderman (1985), (6) Yu and Finlayson (1984b), (7) Kabi, (8) Hanson and Ballard (1968), (9) FDA specification.

cesses now include diafiltration at pH ≈5 with added NaCl to remove most of the aluminum. Although not present in Fraction V (USP), sodium azide, 0.1%, is often added to albumin solutions for laboratory use. It can frustrate the investigator through its inhibitory action on peroxidases used in some applications. Bovine

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Bovine albumin preparations are generally more pure than are those of human albumin; they lack the yellowish color usually seen with HSA solutions.

2. Macromolecular Substances

Macromolecular impurities in Fraction V have been reported sporadically owing to interference with some specific application; they have seldom been quantitated and no doubt vary from lot to lot. Fraction V may legally contain as much as 4% of globulins; normally it contains less than 2%, chiefly α_1-globulins such as orosomucoid.

Enzymatic activities of prolidase (Ganapathy *et al.*, 1982), hexosaminidase (Chen *et al.*, 1991), nucleases (Trujillo *et al.*, 1990), phospholipase A_2 (Elsbach and Pettis, 1973), and a leukocytic protease (Wilson and Foster, 1971) have been noted. Alkaline phosphatase activity is commonly found with albumin of placental origin (Bark, 1971) but is insignificant clinically. Hypotensive activity has been attributed to prekallikrein activator in plasma protein fraction but not in Fraction V (Finlayson, 1980).

Soluble HLA class I antigen was measurable in the range of 2–10 ng/mL in albumin from three of six producers tested (normal serum contains 1328 ± 954 ng/ml) (Santoso *et al.*, 1992). Growth factors possibly detected are epidermal growth factor (Nicholas *et al.*, 1988), erythropoietin (Congote, 1987), and interleukin-1 (Sato, 1987). In one study in Japan, HSA from two manufacturers, but not from five others, showed immune cross-reactivity that was traced to a small amount of contaminating BSA (Murozuka *et al.*, 1990); this was apparently a rare event that arose during manufacture.

Clinical reactions to HSA Fraction V are extremely rare, less than 1 in 32,000 bottles (Turner *et al.*, 1987). Some appeared as chills and fever, which may have been due to bacterial contamination during use (Finlayson, 1980). An antibody against caprylate-treated albumin was found in the blood of one patient (Golde *et al.*, 1971). In another instance, a patient who had shown an anaphylactic reaction to HSA during plasma exchange for myasthenia gravis had no further adverse reactions in five subsequent exchanges conducted with premedication with diphenhydramine (Edelman *et al.*, 1985).

Lot-to-lot variations in albumin performance in various applications have suggested differences due to unidentified constituents. The applications include iodination for tracer studies (Federighi *et al.*, 1966), metabolic turnover measurement *in vivo* (Wallevik and Mouridsen, 1973), hemagglutination testing (King *et al.*, 1991), dye binding (Farrance *et al.*, 1978), CD measurement with and without bound drugs (Perrin and Wallner, 1975), and lipoprotein lipase assay (Whayne and Felts, 1972). A perplexed investigator of the action of heparin preparations once went so far as to declare that crude HSA Fraction V solutions should be labeled "Suitable for Human Use Only—Not for Investiga-

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tional Use" (Lippman and Mathews, 1977) (with appreciation to E. Young, Hamilton, Ontario).

3. Purification to Remove Unwanted Components

Diafiltration, deionization through resin columns, and reworking of the Fraction V precipitate by reprecipitation are employed to reduce the level of low molecular weight components. Defatting by organic extraction and/or charcoal treatment at low pH is used to remove LCFAs and many other lipophilic compounds. Many such procedures are proprietary, such as the preparation of low-folate and low-vitamin B_{12} BSA for culture and testing systems.

Crystallization, although not the epitome of purification, is still a beneficial step in preparing albumin freed of many macro- and micromolecular impurities. The user should be alert for the possible presence of decanol or other aids to crystallization. Many substances such as endotoxins are avoided simply by careful selection of lots of plasma prior to fractionation of albumin; proteases can be avoided by attention to the processing step in which plasma is removed from white blood cells.

Chromatography and gel filtration offer the most refined purifications. They are discussed in the following section.

B. Forms of Albumin Observed

The heterogeneity of "pure" preparations of albumin has attracted the attention of many investigators, in particular the late J.F. Foster. He applied the term microheterogeneity (Foster *et al.*, 1965) to indicate that the differences were often seen only with sophisticated techniques, such as reversible boundary spreading, and were probably minor ones, affecting only local regions or single amino acid residues of the molecule. The causes of many of the differences are now known—among them are polymerization, variant sequences (Table 4-8), varying content of LCFAs and other tightly bound ligands, modifications at the single thiol group, CySH-34 (Chapter 2, Section II,B,5), and covalent changes occurring in the circulation, the latter having been discussed in Chapter 5 (Section II,D). Two reviews of the earlier studies on this subject are those of Janatová (1974) and Foster (1977).

1. Polymeric Forms

Commercial albumin preparations invariably contain a proportion of dimers and higher oligomers (Fig.7-1). They are generally attributed to the pasteurization or other heating steps, which are known to promote polymer formation (Chapter 2,

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Section II,C,2,c). In 34 lots of Fraction V from 22 manufacturers studied by size-exclusion HPLC, dimer content was 1.4–15.4%, polymers 0–9.2%, and monomer 83.8–98.6% (Van Liederkerke *et al.*, 1991); a smaller study found 86–91% monomer in three preparations (Sogami *et al.*, 1984). Lyophilized powders typically accumulate polymers, their amount increasing with storage temperature and moisture content of the powder (Moreira *et al.*, 1992).

Dengler *et al.* (1989) isolated the small amount of high-polymer "aggregate" fraction found after pasteurization. Somewhat amazingly, they found it to contain only 30–50% albumin; both haptoglobin and transferrin were detectable by immunochemical tests, and they proposed that the aggregates were largely complexes of albumin and the heat-denatured forms of some other plasma proteins created by disulfide exchange. Recently hemopexin was also demonstrated in pasteurization induced aggregates, along with traces of DBP and β_2-glycoprotein; removal of these glycoproteins by concanavalin A affinity chromatography prior to heating was proposed (Jensen *et al.*, 1994).

2. LCFA- and Cystine-Related Forms

These two causes of heterogeneity are considered jointly because they are interrelated in their effects.

Total fatty acids are found in sizeable amounts in Fraction V—5.1, 8.1, and 9.7 M/M HSA in one study (Birkett *et al.*, 1978). The bulk of these was caprylate, however, added in pasteurization at 5.4 M/M (Table 7-3). LCFAs are more commonly found at <1 M/M *in toto;* Table 7-4 lists two studies of the LCFA content of HSA and BSA preparations. Because the total in the circulation is 1–2 M/M (Chapter 3, Section I,A), 50% or more is lost during fractionation. Morganthaler *et al.* (1980) measured the loss of LCFAs at different steps of the abbreviated Kistler and Nitschmann procedure. They found 30% loss in the 19% ethanol precipitate, 20% loss in the 40% ethanol supernatant, and an additional 10% loss on the filter, for a total loss of 60%. The distribution of different LCFAs on the Fraction V product is essentially the same as that in plasma, so that a preferential loss of certain fatty acids is not evident. There was no loss of LCFA on albumin isolated in 2.05 *M* ammonium sulfate.

Thiol content of freshly isolated Fraction V albumin is about 0.7 M/M, decreasing to ≈0.3 M/M with storage (Simpson and Saroff, 1958; Janatová, 1974). The breakdown of the attendant heterogeneity has been most elegantly demonstrated by anion-exchange chromatography, notably by the study by Noel and Hunter (1972). Their classic chromatogram of crystalline BSA appears in Fig. 7-3. Earlier studies of BSA (Hartley *et al.*, 1962; Janatová *et al.*, 1968a) and HSA (Janatová *et al.*, 1968b) gave essentially similar results, and more recently an HPLC system has been developed for the separation of mercaptalbumin (Era *et al.*, 1988). Anderson (1966) has studied the thiol-related monomers and oligomers of BSA.

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TABLE 7-4
Long-Chain Fatty Acids Found in Albumin Preparations

Fatty acid	M/M albumin	
	HSA Fraction V[a]	Crystalline BSA[b]
C14:0	—	0.01
C16:0	0.13	0.14
C16:1	—	0.02
C18:0	0.02	0.13
C18:1	0.19	0.22
C18:2	0.12	0.07
C18:3		0.01
C20:0	0.01	0.003
C20:4	—	0.008
Total	0.51	0.62

[a]From Morgenthaler *et al.* (1980) and Rosseneu-Motreff *et al.* (1970).

[b]From Noel and Hunter (1972).

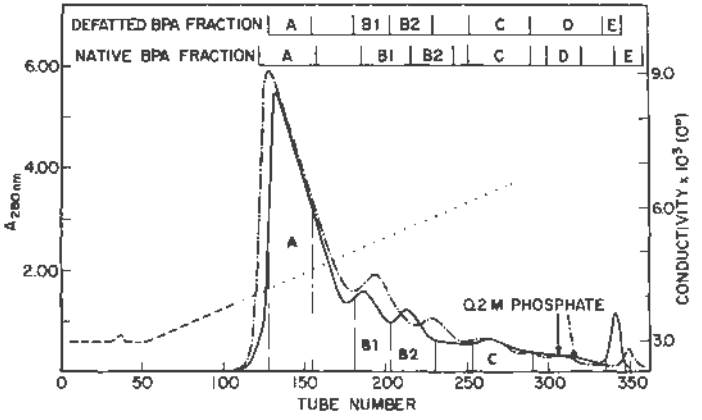


Fig. 7-3. Absorbance of elution profiles of native (solid curve) and defatted (broken curve) BSA on DEAE-Sephadex A-50. The dotted line is conductivity of the eluate. Reproduced from Noel and Hunter (1972) by permission of The American Society for Biochemistry & Molecular Biology. See Table 7-5 for data on the various fractions.

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The fractions of Fig. 7-3 are described in Table 7-5. The leading and major component is mercaptalbumin, with 1 SH/SA. The next peak, B1, was readily convertible to mercaptalbumin by addition of a thiol compound and release of free cysteine; hence it was the half-Cys mixed disulfide. Peak B2 was not readily reducible and its sulfhydryl group is presumably oxidized to a sulfone or a sulfoxide; with HSA Fraction V as starting material a small amount of haptoglobin is identifiable in this region (Janatová *et al.*, 1968b). The small peak C contained 50% of another nonmercaptalbumin monomer form, which was convertible on reduction to yield mercaptalbumin with 0.84 M/M sulfhydryl, and 50% of a dimer. The final peak E, obtained on stripping the column with 0.2 *M* phosphate, contained dimers without sulfhydryls, trimers, and higher polymers; about half of the dimer could be converted to monomer on reduction, so was apparently the S–S form. With BSA freshly isolated under mild conditions the small oxidized nonmercaptalbumin peak B2 was still present, and is believed to exist in serum; the final peak E was absent (Janatová *et al.*, 1980), as were aggregates observable in commercial preparations on gel filtration (Andersson, 1966).

The 0.98 M/M of sulfhydryl in mercaptalbumin is only measurable promptly after isolation; if the protein is kept at pH 7, it quickly drops to ≈0.8 M/M.

The variation in LCFA content of the fractions is of interest (Table 7-5). Although the starting BSA had 0.48 M/M LCFA, the mercaptalbumin contained less than half of this, 0.22 M/M. The nonmercaptalbumin forms, B1, B2, and C, each contained ≈1 M/M. The inverse relationship between sulfhydryl and LCFA levels in the eluted fractions (Table 7-5) recalls the effect of bound LCFAs in widening the cleft harboring CySH-34 (Chapter 2, Section II,B,5) to allow greater accessibility to oxygen. Unsaturated LCFAs, C18:1 and C18:2, are particularly effective (Chapter 3, Section I,A,3), perhaps through a peroxidative action. Oleate is the chief fatty acid missing from mercaptalbumin (fraction A), and addition of linoleate was found to cause a drop in sulfhydryl content from 0.9 to 0.76 M/M in 2 weeks (Noel and Hunter, 1972).

Another technique to identify microheterogeneity of albumins is the solubility profile in 3 *M* KCl in the pH range 4.5–3.5 (Sogami and Foster, 1968). Still another is differences in the susceptibility of S–S bonds to reduction in different fractions, demonstrated by Habeeb (1968) and Sogami *et al.* (1969).

Isoelectric focusing detects several fractions. The migration of LCFAs to yield fat-free and highly fatted, ≈6 M/M, forms in this technique was discussed in Chapter 3 (Section I,A,2). Binding of ampholyte molecules can be a source of artifact (Wallevik, 1973a). Spencer and King (1971) found widespread microheterogeneity of BSA monomers, both commercial crystalline forms and freshly prepared albumin with the thiol blocked with cysteine, on isoelectric focusing in 6 *M* urea in a gel. They raised the possibility that the heterogeneous forms arose from modifications *in vivo* such as those discussed in Chapter 5 (Section II,D).

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TABLE 7-5
Identification and Fatty Acid and Sulfhydryl Content of Fractions of Fig. 7-3[a]

Fraction	Identification	LCFA[b]	Thiol[b]
A	Mercaptalbumin monomer	0.22	0.98
B1	Nonmercaptalbumin, convertible	0.97	0.16
B2	Nonmercaptalbumin, oxidized	0.99	0.06
C	Nonmercaptalbumin monomer + sulfhydryl-containing dimer	1.04	0.36
E	Sulfhydryl-free dimer + higher polymers	0.57	0.14
	Calculated average starting material	0.48	0.65

[a]Data for native crystalline BSA elution from DEAE-Sephadex A 50 (Fig. 7-3); extracted from Noel and Hunter (1972).
[b]Data in mole/mole albumin.

III. ALBUMIN PRODUCTS AND THEIR *in Vitro* APPLICATIONS

The number of albumin products listed in laboratory supply catalogs bewilders the buyer. Many grades of purity or pretreatment for some special application are available, and judgment is needed to select a product for a specific purpose. When the albumin is intended simply to protect an enzyme from being adsorbed on the surface of a container, isolation by heat-shock treatment would not be harmful and may even be beneficial, but it could be ruinous to experiments to measure ligand binding or configuration of the albumin; in this case crystalline albumin or albumin isolated by chromatography would be indicated. Manufacturers recommend that, for a new application, a user start with a very pure preparation, such as crystalline BSA, and then attempt to substitute a less expensive form if this can be done without effect on the system.

A. Products

1. Species

Serum albumins of 19 animal species are listed in the 1994 Sigma Chemical Company catalog: baboon, cat, cow, chicken, dog, donkey, goat, guinea pig, hamster, horse, human, monkey, mouse, pig, pigeon, rabbit, rat, sheep, and turkey. All are available as Fraction V, and 11 species are also marketed in more purified forms such as fat-free or "essentially globulin-free"; human,

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bovine, and rabbit albumins can be purchased in crystalline form. In the author's experience the Fraction V products of many animals may not be as pure as those of humans and the cow, a not-surprising finding inasmuch as the Cohn fractionation was devised around the plasma of these two species. Users are advised to check Fraction V albumins of other species by electrophoresis and gel filtration for the presence of other proteins or of albumin breakdown products.

Although HSA is the leading product in terms of quantity, most of it serves the *in vivo* clinical market (Chapter 6, Section III); only 11 HSA products are listed compared to 37 for BSA. Of the latter, 27 are powders and 10 are solutions, ranging in concentration from 7% (70 g/L) to 30 or 35% (350 g/L). This large menu reflects the much greater use of BSA than of HSA in laboratory and industrial operations.

Although more than 20 firms produce HSA for clinical use, only about 7 United States firms produce BSA. Hepatitis and immunodeficiency viruses are not a concern with BSA, but a significant obstacle is the slow virus or perhaps a prion (small infective protein), which causes bovine spongiform encephalopathy (BSE), or "mad cow disease," particularly in Great Britain. This agent is heat stable and must be avoided in selecting the bovine plasma; the virus of blue tongue disease, which can be transferred from cows to sheep, on the other hand, is heat labile and can be inactivated in processing.

On a lighter vein, one concern with BSA products seems overdone. The Material Safety Data Sheet (MSDS) required by the U.S. Occupational Safety and Health Administration (OSHA) warned of a "Fire and Explosion Hazard" of a vial containing 200 mg of BSA powder (Doumas, 1994)!

2. *Purity*

Crystallized (1–3×) albumins are recommended for the highest purity in terms of absence of globulins (<1%) and low molecular weight contaminants. Ash content is typically <0.5%. The user should inquire whether the product had been pasteurized, which is now the customary commercial practice, at any stage. Also to be recommended is a BSA product made by combined salt fractionation, ion-exchange chromatography, and gel filtration, which avoids the rigors of the cold-alcohol procedure.

Most preparations contain polymer forms, those purified by gel filtration having the least. The highest monomer content, 99%, is found with BSA having its single sulfhydryl blocked by cysteine. Pure mercaptalbumin does not appear to be available; the tendency for the thiol to dimerize or undergo oxidation, cited above, is responsible. Other products are actually deliberately made to contain high proportions of albumin polymers to cause high avidity in hemagglutination systems.

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Cleaned-up Fraction V preparations are made in several ways. Treatment with charcoal and extensive dialysis or diafiltration removes most of the low molecular weight affiliates. Acidification prior to charcoal treatment is the standard method to prepare low-fatty acid forms of albumin, starting either with Fraction V or crystallized material; extraction with organic solvents such as ethanol or acetone is employed, although the conditions are proprietary information. A similar extraction removes endotoxin and most lipid soluble hormones, which are undesirable in many culture systems. Another BSA product has been defatted and palmitate added to >3 M/M.

Traces of active enzymes such as proteases and nucleases are difficult to remove entirely. The commercial preparations, which are billed as low in these constituents, are generally obtained by rigorous selection of the source plasma, as noted above.

The factors possibly affecting cell culture *in vitro* are so numerous and unclear that albumin production batches are actually tested in critical systems prior to release. This applies to culture of bacteria, mammalian cells, and sensitive organisms such as *Leptospira*, *Treponema*, and *Mycobacteria*.

3. Derivatives

Albumin modified by acetylation has been found to be more effective than native albumin in protecting the enzymes in the PCR technique. Its higher net negative charge may be responsible, causing a greater affinity for membranes, surfaces, and RNase, and one wonders if acetylated albumin might not have wider uses in protecting macromolecules. Carboxymethyl and methylated derivatives are also available; the latter are useful in chromatography of nucleic acids.

Albumin, particularly BSA, has been popular as a macromolecular carrier for haptens and for reporter compounds in histochemical studies. Commercial manufacturers have lightened the burden on researchers by making available BSA modified by addition of simple sugars, among them maltosyl, lactosyl, cellobiosyl, melibiosyl, glucosamide, galactosamide, and fucosylamide groups. Dinitrophenyl (DNP)-HSA is available for use as an antigen. Glycated BSA and HSA are marketed; these are apparently prepared by nonenzymatic glycosylation *in vitro*, and caution is needed in equating them to the forms produced *in vivo*.

For measuring protease action, BSA coupled to dyes, azoalbumin and naphthalene blue black albumin, provides a useful substrate. Other reporter groups on BSA available from leading biochemical supply houses include biotin, fluorescein, rhodamine, resorufin, *p*-aminophenyl, and colloidal gold plus biotin. These are generally coupled to the albumin via sugar groups such as glucopyranosyl, mannopyranosyl, galactopyranosyl, lactopyranosyl, fucopyranosyl, and *N*-acetylgalactosaminide.

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B. *In Vitro* Applications

BSA predominates here, HSA being employed *in vitro* only when a human protein is specifically required to avoid allergic or other types of reactions. Manufacturers are generally well attuned to the particular requirements of albumin users, and provide tables showing the most suitable albumin product for different applications. Reference to these tables or consultation with the producer is a wise step before ordering.

1. Cell Culture

Tissue culture historically used diluted plasma as the growth medium, and plasma or fetal calf serum is still employed for many systems. Biologists continually seek a more defined medium, however, in which to study factors controlling cell growth. The ability of purified serum albumin to replace complex mixtures was recognized by Jacquez and Barry in 1951; Barnes and Sato (1980) and Iscover *et al.* (1980) were leaders in the subsequent formulation of serum-free media. These usually contain transferrin and small amounts of insulin as well as albumin. The albumin products should optimally be those certified as nondenatured, endotoxin and *Mycoplasma* free, generally fat free, and optimally pretested for support of the tissue under study.

Several bacterial growth media benefit from addition of BSA Fraction V. The Barbour–Stoenner–Kelly medium for detecting *Borrelia* is an example (Callister *et al.*, 1990); supplementing yeast agar extract with BSA for growing *Legionella* is another. Among somewhat higher organisms, fat-free BSA was found capable of replacing plasma in growing the malarial parasite, *Plasmodium falciparum* (Siddiqui and Richmond-Crum, 1977); larvae of both the canine hookworm, *Ancylostoma caninum* (Hawdon and Schad, 1991), and the mosquito, *Brugia malayi* (Nayar *et al.*, 1991), will continue to develop *in vitro* with BSA in place of serum.

There is significant interest in growing blood cells, especially lymphocytes, *in vitro*. HSA, 1%, will substitute for serum in promoting lymphoblast replication (Drouin *et al.*, 1987), and BSA or HSA is useful with human B lymphocytes (Wasik *et al.*, 1987) and mouse BALB/c 3T3 cells (Bedotti *et al.*, 1990). Granulocytes (Ieki *et al.*, 1990), bone marrow cells (Agarwal and Chauhan, 1993), and myeloid leukemia cells (Kiss, 1990) are other applications. With erythropoietic cells (Akahane *et al.*, 1987) and erythropoietin-dependent lymphocytes BSA can serve as the only protein necessary for colony formation and growth.

Hepatocytes are cultured for investigative purposes and for potential clinical use as a liver replacement. Addition of HSA extends the survival of fetal rat liver cells (Hellmann *et al.*, 1990). Proximal tubular epithelium cells grow for long periods in BSA, insulin, and epidermal growth factor in a defined medium

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designed to test renal carcinogenicity (Hatzinger and Stevens, 1989). Isolated rat pancreatic islet β-cells show 75% or more survival with 1% BSA in place of fetal calf serum (Ling *et al.*, 1994). Rat fibroblasts (Okuda and Kimura, 1988) and human muscle satellite cells (Ham *et al.*, 1988) will grow or metabolize in a BSA-containing medium.

Tracheal epithelial cells are an example of the need to check the type of albumin product to be used; they show a consistent response of BSA prepared by heat shock followed by dialysis, charcoal treatment, and deionization, but a variable response to BSA Fraction V (Thomassen, 1989). This effect is probably caused by nutrients or hormones in the cruder preparation. Lysophospholipids bound to albumin were shown to cause neurite retraction in pheochromocytoma cells (Tigyi and Miledi, 1992), and bound LCFAs to stimulate steroid production in Leydig cells (Melsen *et al.*, 1991).

A transport activity of albumins (Chapter 5, Section II,B) is apparent in several culture systems. Schneider (1989) found that growth and monoclonal antibody production by human hybridoma cells are optimal with a trace, 30 mg/L, of BSA carrying linolenic acid. A reverse effect is seen in the prevention of the inhibitory effect of polyunsaturated LCFAs on human hepatoma cells by addition of 0.5% fat-free albumin (Hostmark and Lystad, 1992). Fat-free BSA, 2%, was stimulatory to lactate oxidation and lipogenesis in rat neurons and astrocytes in primary culture (Vicario *et al.*, 1993), and was effective in supplying retinol to human retinal epithelial cells for its esterification to retinyl palmitate (Das and Gouras, 1988).

Surface-active effects are important for cell growth. Precoating with albumin markedly increased cell spreading in monolayer cultures on glass (Kieler *et al.*, 1989) or several types of polymeric materials (Schakenraad *et al.*, 1989). Sertoli cells showed maximal transferrin secretion on Millipore cellulose ester filter supports presaturated with either fetal calf serum or BSA (Janecki and Steinberger, 1988). Attachment and growth of epithelial cells from human colonic biopsies was optimal on a mixture of BSA and collagen (Buset *et al.*, 1987). Grinnell and Backman (1991) postulated that spreading of cells on an albumin monolayer depended on an interaction of manganese and the membrane protein, integrin, in their system.

In lung perfusion systems 4.5% albumin will substitute for plasma (Kröll *et al.*, 1990). It also is effective, mixed with dimethyl sulfoxide and human serum, for cryopreservation of human bone marrow (Reiners *et al.*, 1987a) or hematopoietic stem cells (Reiners *et al.*, 1987b).

Reproductive cells have special requirements when maintained *in vitro*. Inclusion of albumin in the culture medium enables "capacitation" of human sperm—motility and ability to penetrate the zona pellucida of the ovum (Rosselli *et al.*, 1990). For *in vitro* fertilization and subsequent embryo culture, HSA is a safe and effective substitute for serum (Holst *et al.*, 1990; Khan *et al.*, 1991). Note that BSA should not be used for such procedures, or in rinsing flu-

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ids for follicle aspiration; allergic reactions against BSA have occurred on several occasions (Moneret-Vautrin *et al.*, 1991; Gamboa *et al.*, 1992). One wonders if the use of BSA in manufacture of cosmetics, as advertised by one manufacturer, might not lead to skin rashes on a similar basis.

2. Cell Separations

Density gradient centrifugation is the most important technique for separation of cells, although electronic cell sorting is growing in utility. Albumin was first suggested as the density-forming solute by a resourceful colleague of mine, J.W. Ferrebee, and Q.M. Geiman in 1946, and has been widely used ever since. Harwood reviewed density gradient separations in 1974. More recently Chadwick *et al.* (1993) have studied the interrelation of temperature and osmolality control with BSA to avoid cellular swelling, and albumin, metrizamide, and dextran-40 have been found to be the only effective materials of seven tested for isolating islets (van Suylichem *et al.*, 1990). The digestion of pancreas pieces with collagenase prior to isolation of islet cells was improved in the presence of 10% BSA (Wolters *et al.*, 1990).

With guinea pig megakaryocytes, subpopulations can be obtained by sedimentation without centrifugation (Schick *et al.*, 1989). Viable microvessels of brain could be separated with BSA whereas when sucrose was used they were inactive (Sussman *et al.*, 1988). Porcine spermatozoa with increased resistance to frozen storage have been isolated in a 4%, 10% discontinuous BSA gradient (Estienne *et al.*, 1989); earlier attempts to separate X and Y chromosome sperm do not appear to have been productive (Dmowski *et al.*, 1979).

3. Protection of Macromolecules

An important use of albumin in the laboratory is to prevent loss or inactivation of delicate proteins or nucleic acids by acting as a surrogate macromolecule that binds to surfaces of vessels and pipettes. As little as 1 mg/mL of BSA is usually sufficient. Enzyme isolations, immunoassays using radiolabeled reagents, or assays with low concentrations of antigens and antibodies all benefit from the presence of albumin. A related use is to block unfilled areas of films after transferring proteins or nucleic acids in Northern, Southern, or Western blots. For these purposes a protease- and usually a nuclease-free albumin is necessary to prevent damage to the test molecules. The high-avidity (high polymer content) products are suggested as being particularly effective blocking agents. Azide as preservative must be avoided if peroxidases are to be part of the system.

4. Applications in Immunology and Hematology

Albumin is frequently used as a model antigen, as described in Chapter 3 (Section IV). It is also widely employed as a vehicle for haptens; DNP–HSA,

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for example, is used to test allergic responses leading to release of histamine by mast cells (Levi-Schaffer *et al.*, 1990). In many instances BSA is the carrier for antibody generation for immunoassays for hormones, other metabolites, or drugs. Coupling of bacterial toxins (Newman, 1990) and virus particles (Martin *et al.*, 1988) with rabbit albumin allowed generation of soluble antibodies in rabbits without adverse reactions from the antigens.

As a diluent in blood banking or immunohematology testing, a protease-free, native, fat-free BSA preparation is recommended. For systems using hemagglutination the high-avidity products are desirable.

5. *Applications of Immobilized Albumin*

In addition to the use of methylated albumin in the chromatography of nucleic acids, the ligand-binding ability of native albumin can be employed to perform selective separations of complex biological compounds. Lipoteichoic acid of *Streptococcus pyogenes*, which binds to the LCFA site, can be isolated in active form from human albumin coupled to agarose (Simpson *et al.*, 1980). Human interferon will bind to HSA-agaroses and will be retained in 1 *M* NaCl, but will elute with a polarity-reducing agent such as ethylene glycol (Huang *et al.*, 1974) (see Chapter 3, Section I.E.5).

Stereoselective separations and reactions have potential commercial utility. Albumin bound to agarose or immobilized on silica with, for example, glutaraldehyde (Andersson *et al.*, 1992) is employed. Only the L enantiomer of tryptophan is bound (Yang and Hage, 1993), and one of the chiral forms of warfarin (Lagercrantz *et al.*, 1981). Racemic mixtures of aryl propionate antiinflammatory drugs (Noctor *et al.*, 1991) and N-methylated barbiturates (Krug *et al.*, 1994) can be resolved. Recently the application of immobilized albumin has been extended to affinity capillary electrophoresis (Birnbaum and Nilsson, 1992; Arai *et al.*, 1994).

The semicatalytic effect of albumin in promoting chemical changes in substances such as eicosanoids and some drugs (Chapter 5, Section II.B.4) has been under study as a means of carrying out chirally specific reactions. Many reactions have been tested—examples are preferential hydrolysis of D-enantiomers of aromatic acetates (Kurono *et al.*, 1983b), stereoselective oxidation of aromatic sulfides and sulfoxids (Sugimoto *et al.*, 1981), and the synthesis of optically active epoxynaphthoquinones (Colonna *et al.*, 1988).

6. *Primary Protein Standard*

The use of pure HSA as a standard for clinical albumin assays was considered in Chapter 6 (Section I.B). This section will examine the use of albumin, particularly BSA, in assays for total protein.

Nearly all analyses for protein, whether by the Lowry, Coomassie Blue, bicinchoninic acid, or biuret reaction, refer their results to BSA as the standard.

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Not only is BSA the purest protein available commercially at reasonable cost, but its freedom from carbohydrate and other prosthetic groups makes it an ideal reference for the biuret procedure, which measures chiefly the content of peptide bonds. BSA products used as standards should be of highest purity—99% albumin rather than the ≥96% of Fraction V. BSA standards should be purchased in solution form; few laboratories are equipped or experienced in preparing accurate protein standards from powders, which may contain 5–15% moisture as well as ash and other unknown substances. (For preparation of albumin solutions from powders, see Section IV.)

A 70-g/L, sulfhydryl-blocked monomeric BSA is recommended because it will not tend to accumulate polymers. A similar preparation (Peters, 1968), listed as Standard Reference Material (SRM) 927, is distributed by the National Institute for Standards and Technology in Gaithersburg, Maryland, and has maintained its stability for over 10 years. It is commonly used as the primary reference for preparing calibrating solutions for clinical total protein assays.

Albumin is widely used as a 66.5-kDa molecular weight standard in SDS-gel electrophoresis; again the monomer is recommended unless presence of oligomers is actually desired. HSA has been prescribed as the carrier for bilirubin in a candidate reference method to measure bilirubin (Vink *et al.*, 1987), and as the host for haptenlike compounds in assays for protein-bound fructosamine (Podzorski and Wells, 1989) or sialyllacto-*N*-fucopentaose, a standard for tumor-associated antigens (Masson *et al.*, 1990). In immunochemical blotting techniques, BSA is frequently employed as a test antigen to demonstrate the validity of the assay (Leong and Fox, 1988).

IV. TESTED PROCEDURES FOR USE IN LABORATORY

The procedures and recommendations that follow evolved from experience in our laboratory for at least 30 years. The assistance of R.G. Reed and C.M. Burrington of that laboratory is much appreciated.

A few general principles of good protein chemistry must be respected. Change but one parameter at a time in a separation step: pH, ionic strength, temperature, etc. Change conditions by adding reagents slowly—dropwise or in a capillary stream. Provide prompt, complete, but *gentle* mixing. Use low temperatures. Maintain the pH of the albumin near neutrality, between pH 5 and pH 8, and the salt concentration above ≈0.02 *M* to avoid possible disulfide shuffling. Keep the protein concentration moderately high, 10 g/L or more, to avoid losses or denaturation on surfaces, yet not so high as to foster polymer formation. In adding ethanol, predilute it to <80% (v/v) to avoid generation of its considerable heat of dilution in the presence of the proteins; allow time for "conditioning" or "maturation" of precipitates to minimize entrapment or adsorption (More and Harvey, 1991).

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A. Preparing Solutions of Albumin

Considering the plethora of commercial albumin products available, authors should state the precise nature of the albumin preparation they used. Simply stating "Sigma A1887" does not help the reader; that catalog number may be retired, and the user should know the prior treatment of the albumin, fatty acid content, state of the thiol group, percent purity, and content of oligomers.

For consistent performance of successive lots, the albumin should be purchased directly from its manufacturer rather than through a supplier. Supply houses do not usually acknowledge the source of their products, and the next purchase may come from a different source.

Dissolving a protein can often be the most frustrating operation of the day if it does not proceed rapidly, and protein chemists learn to prepare solutions the night before, if feasible. The presence of some salt, even 0.02 *M* NaCl, speeds dissolution; to adjust the pH it is helpful to note that 110 μmol of NaOH will raise the pH of a solution containing 1 g of albumin by ≈1 pH unit in the pH range 5.5–7.5. Making solutions of proteins to volume in a volumetric flask is hindered by inevitable foaming, and the following approach can be used instead to make solutions of any concentration in any total volume desired.

Weigh the amount of albumin powder desired; allow 10% for moisture, unless you have determined the moisture by drying to constant weight *in vacuo* at 105° (the albumin thus dried must be discarded), and 1% for salts and other material. Calculate the weight of albumin, obtain its volume in solution by multiplying by the partial specific volume of 0.73 mL/g (Table 2-3), and add water or buffer calculated to achieve the desired total volume. As an example, 100 mg of BSA powder with 10% moisture and 1% salts would equal 89 mg of protein, occupying 65 μL, plus 10 μL of water. Adding 1.705 mL of solvent would give a total volume of 1.780 mL with an albumin content of 50 mg/mL.

The concentration should be checked, most conveniently by reading $A_{279\ nm}$ and using the absorbance at 1 mg/mL of 0.531 for HSA or 0.667 for BSA (Table 2-3). Less than pure preparations will have higher absorbances, and their concentrations can be assayed by a biuret procedure. Using the reagent described by Doumas *et al.* (1981), 3 g/L $CuSO_4 \cdot 5H_2O$, 9 g/L tartrate, $KNaC_4H_4O_6 \cdot 4H_2O$, 5 g/L KI, in 0.6 *M* NaOH, we found the A_{540nm} of a 1 mg/L solution of BSA to be 0.296; a commercial standard BSA solution should be used for comparison.

B. Characterizing Albumin Preparations

A highly pure preparation of BSA or HSA should show an absorption minimum near 255 nm, and the ratio A_{255}/A_{280} should be ≤0.5. A faint turbidity is a usual cause of a higher ratio.

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Homogeneity of the albumin may be assessed by electrophoresis using polyacrylamide gel or capillary electrophoresis at pH 8.6 on the undenatured albumin. Highly recommended as a quick and convenient procedure is electrophoresis on cellulose acetate strips; the Helena Co. (Beaumont, Texas) markets a useful apparatus. The strips, fixed with TCA and stained with Ponceau Red, show both purity of the albumin and identity of other components by their mobility in less than 30 min. This technique requires only 8 µL of sample, which can be as dilute as 1 mg/mL, and is particularly useful in identifying fractions from a chromatographic separation or judging the progress of a digestion. It is easily adaptable to observing binding of radiolabeled ligands (Fig. 4-5) or identification of bands by immunofixation. By testing at several conditions of pH (e.g., pH 5.0, 6.5, and 8.6) components with similar mobilities at one pH may be differentiated at another.

Assay for Thiol Groups. The DTNB method based on Janatová *et al.* (1968a) is used.

Reagents:
- (Reagent A) 396 mg DTNB (5,5'-dithiobis(2-nitrobenzoic acid) in 100 ml Reagent B (= 0.01 *M* DTNB).
- (Reagent B) 0.037 *M* Na-PO_4 buffer, pH 8.00.
- (Reagent C) 0.0074 *M* Na-PO_4 buffer, pH 8.10.
- (Reagent D) 0.1 *M* EDTA disodium, pH 7.0.

Specimen: 0.8 ml of Reagent C containing 1–2 mg albumin, pH adjusted to 7.0.

Procedure: Add 10 µL of Reagent D and 0.2 mL Reagent A; mix immediately. Total volume = 1.01 mL. Allow to sit 30–60 min in the dark at room temperature. Read A_{435nm} against water; subtract readings of blanks without protein and without Reagent A. Calculate m*M* SH by dividing A_{435nm} by 11.3; divide by m*M* albumin to get mol/mol SH. L-Cysteine hydrochloride is a useful standard; it reacts much more quickly than does albumin.

Assay for Carbohydrate. For *total hexose* the Harvard Physical Chemistry Laboratory used the orcinol method based on Sørenson and Haugaard (1933); a pure albumin should contain less than 0.05% total carbohydrate. **Use CARE** in handling the strong sulfuric acid in this procedure. For glucosyl groups the fructosamine assay (Guthrow *et al.*, 1979) is commonly used; normal content of HSA is 1–2% glycoalbumin. For individual amino sugars such as glucosamine and galactosamine, ion-exchange chromatography of acid hydrolyzates is useful.

Assay for Fatty Acids. Unesterified fatty acids are generally extracted with H_2SO_4–methanol (Dole, 1956) and quantified as the copper soap (Evenson and Deutsch, 1978). Individual fatty acids are measured by gas chromatography of methyl esters (Morgenthaler *et al.*, 1980).

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C. Isolating Albumin from Serum

The elegant approach is to prepare albumin from an ammonium sulfate fraction from fresh plasma or serum by ion-exchange chromatography (Fig. 7-3). Dilute serum 1:1 with water and slowly mix in an equal volume of saturated ammonium sulfate at room temperature to give 50% saturation; adjust pH to 6.8. After 4–16 h, centrifuge at 3000 *g*; carefully adjust the supernatant to pH ≈4.4 (this should give maximal precipitation) with 1 *M* acetic acid in 50% saturated ammonium sulfate. Let sit 2 h or more; centrifuge as above. Suspend the precipitate in water and dialyze or wash on an ultrafilter by diafiltration; for this operation concentrate the solution repeatedly on a YM30 ultrafilter (Amicon Corp., Danvers, Massachusetts) with addition of water each time; finally wash into 0.02 *M* $Na\text{-}PO_4$ (sodium phosphate) buffer, pH 7.0.

Prepare a column, ≈100-mL bed volume per g albumin to be applied, of DEAE agarose equilibrated with the 0.02 *M* phosphate buffer. Apply the albumin solution, pump at ≈15 mL/h/100-mL bed volume for 5–6 h; then start a gradient of 300 mL of 0.02 *M* to 300 mL of 0.08 *M* phosphate, pH 7, per 100-mL column volume. See Fig. 7-3 for the expected elution profile; essentially pure mercaptalbumin is in the first major peak.

A simpler procedure is to apply diluted or dialyzed serum to a column of Cibacron Blue F3GA-agarose (Affi-Gel Blue, Bio-Rad Corp., Richmond, California) in 0.1 *M* phosphate buffer, pH 6.8; capacity is about 10 mg albumin/mL of bed, but note that the affinity for Cibacron Blue varies with species (Chapter 3, Section I.C). Albumin is eluted following addition of 2 *M* NaCl, and the column is flushed with 8 *M* urea. Accompanying glycoproteins are then removed from the albumin on passage through a column of concanavalin A (ConA Sepharose, Pharmacia Corp., Piscataway, New Jersey) in phosphate-buffered 0.15 *M* NaCl. Finally the albumin monomer is obtained as the major peak on a column of Sephadex G-100 in the same buffer.

Another simple procedure, although with a risk of unknown structural change, uses TCA–alcohol, and can be applied to extraction of albumin from liver (East *et al.*, 1973). To a washed, 5% TCA precipitate of tissue or serum, add 1% TCA/95% ethanol and stir well at room temperature (lower temperatures allow inclusion of more impurities); solubility is probably 10–20 mg/mL. Filter on a Buchner funnel. To recover albumin from the filtrate either dialyze against water or precipitate by neutralization to pH ≈5 with sodium acetate.

Albumin preparations should be passed through a 0.2-μm ultrafilter and stored at 3° in >1% concentration, at pH 6.5–7 with ≥0.02 *M* salt. If sterility is not maintained, sodium azide, 0.01%, may be added as a preservative, both on storage and to the buffers during isolation.

Locating Albumin in Column Elution Patterns. The presence of protein in fractions collected from column runs may be tested by flicking the tubes; a lasting

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foam at the surface occurs with ≥0.1 mg/mL protein. Albumin may be easily identified by adding 5-μL aliquots to 100-μL portions of BCG reagent in a 96-well plate and noting a blue color appearing within 30 s. The reagent (Doumas *et al.*, 1971) contains 0.15 m*M* (10.5 mg/L) BCG in 0.075 *M* sodium succinate buffer, pH 4.2, 1.3 g/L Brij-35.

D. Modifying Albumin

1. S–S Reduction and Blocking

To remove mixed disulfides from the albumin thiol group (CySH-34), add 0.01 *M* dithiothreitol at pH 6 (do not allow excursions below pH 5 or above pH 7) for a few hours, then remove the low molecular weight compounds on Sephadex G-25 or by diafiltration at the same pH.

To block the free thiol of albumin reversibly (Sogami *et al.*, 1969), add 5 M/M L-cystine, dissolved in a minimum amount of NaOH, keeping the pH in the range 7.5–8 but never >8.0; some cystine may precipitate as a white solid. Remove unbound cystine as above after 2–5 h at room temperature. To alkylate the free thiol, add iodoacetamide, 6 M/M, instead of cystine and proceed as above.

For complete reduction of the 17 disulfide bonds, dissolve at 10 mg/mL in 10 *M* urea, 0.2 *M* Tris-Cl, pH 8.6, 1 m*M* EDTA (Johanson *et al.*, 1981); add a 10-fold excess of dithiothreitol or dithioerythritol (= 25 m*M*). After 4–16 h at 23° isolate the reduced protein by elution from a Sephadex G-25 column in 0.1 *M* acetic acid. Keep below pH 5 to avoid reoxidation. To alkylate all thiols, add an excess (0.1 *M*) of iodoacetamide at the end of the reduction and allow to stand at pH 8.0 in the dark for 1 h; then remove the low molecular weight compounds as above or by dialyzing against 0.1 *M* ammonium bicarbonate at pH 8. Iodoacetamide yields a more soluble alkylation product than does iodoacetic acid.

To *oxidize* the cystine sulfurs, dissolve albumin in 99% formic acid, 40 mg/mL, and add 50 μL of 30% H_2O_2 per mL. After 2 h at 23° rinse into ≈20 volumes of water and lyophilize. Cystine yields cysteic acid and methionine yields the sulfone; if chloride is present chlorotyrosine will form (Hirs, 1956).

2. Fatty Acids

Defatting is easily accomplished by bringing an albumin solution to pH 3 with 0.1 *M* HCl or 0.2 *M* formic acid; released LCFAs appear as a faint turbidity. They are removed by immediate passage over a hydrophobic resin (Scheider and Fuller, 1970) such as XAD-2 (Mallinckrodt) or by adding an equal weight of dextran-coated charcoal (1:20) and centrifuging after 60 min (Chitpatima and Feldhoff, 1983); the solution is then brought to neutral pH.

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Adding LCFAs can be accomplished by drying an alcoholic solution of the fatty acid as a thin film on the walls of a vessel or on diatomaceous earth, then adding a solution of albumin at pH 7.4; binding (maximum 6 M/M) occurs in less than 1 h at 23°.

3. *Bilirubin*

Bilirubin and most other bound substances can be removed by slowly passing a solution of albumin at pH 7.4 over a column of HSA-agarose (Plotz *et al.*, 1974). The bilirubin can be seen as a yellow band at the top of the column.

To *add bilirubin*, dissolve the desired amount of bilirubin (M_4 = 584) in a minimum volume of 5 m*M* NaOH plus 1 m*M* EDTA and add slowly to a solution of albumin buffered at pH 7.4 with good mixing.

4. *Iodination*

Currently used in our laboratory is the Iodo-Gen reagent, 1,3,4,6-tetrachloro-3α,6α-diphenylglycouril (Pierce Chemical Co., Rockford, Illinois; M_r = 432). Dry 100 µg of Iodo-Gen on the surface of a vessel from solution in chloroform or dimethyl sulfoxide; rinse with buffer. Add 1 mg albumin in pH 7.4 buffer, then the $^{125}I^-$ or $^{131}I^-$; carrier ^{127}I may be added but the final product should not exceed 1 atom I/mol albumin. After 15 min remove the solution and add an excess of NaI (0.25 *M*). Remove I^- by gel filtration or deionization. Use **appropriate CARE** in dealing with the radioisotope. Note that the reagent will oxidize the SH group of albumin, on a 4:1 molar ratio (McClard, 1981), so the reagent should at least be present in a 0.25 M/M excess or the thiol group should have been previously blocked.

5. *Measuring Ligand Binding*

The technique will depend on the molecular size and solubility of the ligand. For low molecular weight soluble compounds such as dyes and most drugs, equilibrium dialysis is generally used to measure the free compound. For LCFAs, which will not cross dialysis membranes, equilibration with a heptane layer or with BSA-agarose is useful (see Chapter 3, Section I,A,2). For bilirubin, the change in optical absorbance, CD, or fluorescence may be used, as well as the peroxidase method or the BSA-agarose procedure (see Chapter 3, Section I,B,1).

6. *Albumin Immobilized on Agarose*

Albumin is readily coupled to agarose activated with cyanogen bromide. **CARE should be used** with this noxious reagent. We found that 200 mg of

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CNBr/ml agarose will couple 5–10 mg/mL of albumin. The albumin–agarose may be used as above, or for the isolation of pure antialbumin antibody (Peters and Reed, 1980). The bound antibody can be eluted at pH 2.4 with 0.05 *M* NaH_2PO_4 buffer. To isolate albumin by immunoaffinity on antialbumin–agarose, however, requires stronger conditions, 1 *M* NH_4OH.

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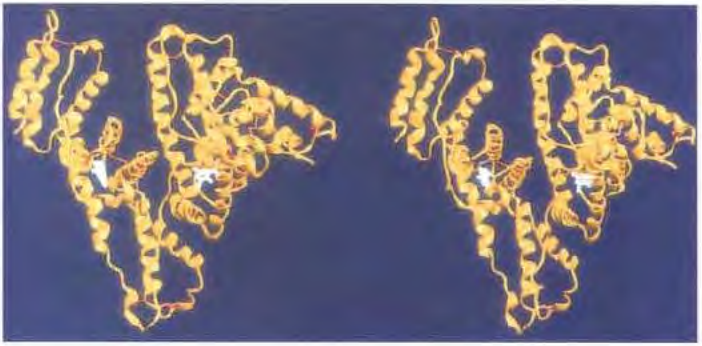


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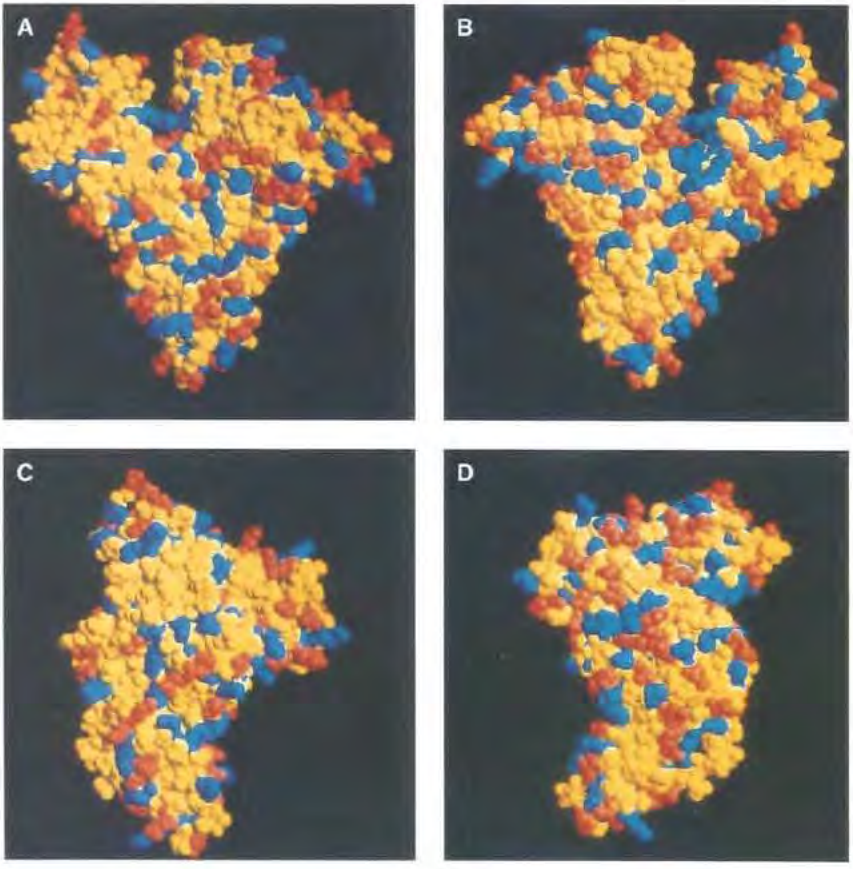


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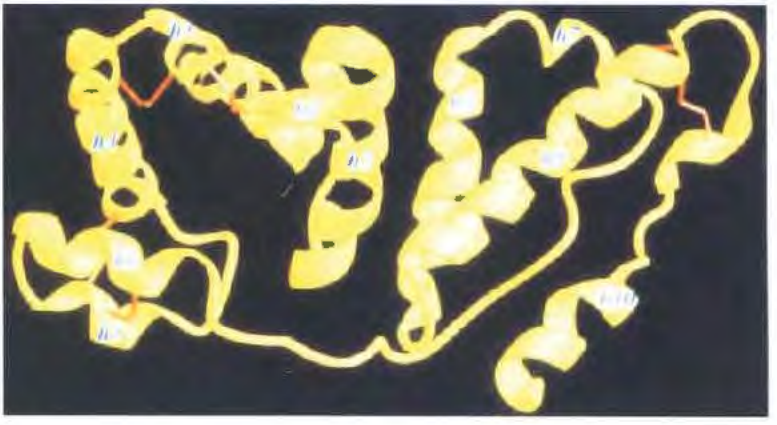


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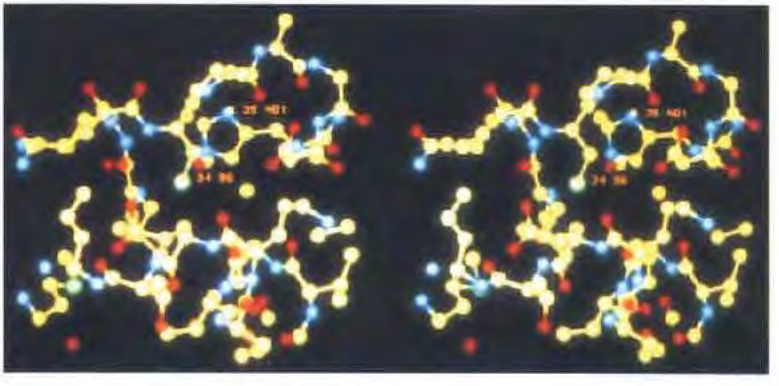


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