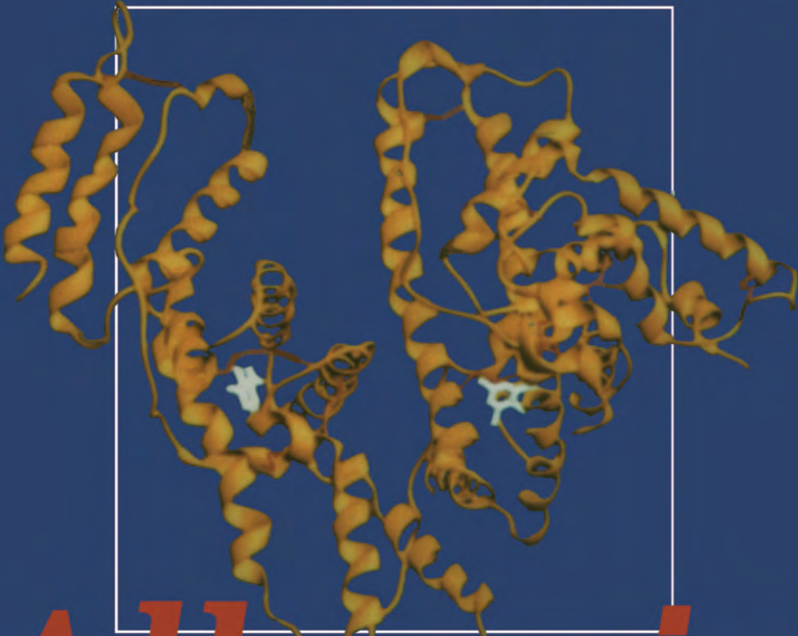


All about



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.

All about Albumin

Biochemistry, Genetics, and Medical Applications

By Theodore Peters, Jr.

Research Institute

The Mary Imogene Bassett Hospital

Cooperstown, New York



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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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Contents

Foreword: All about Albumin	xi
Preface	xv
List of Abbreviations	xix
 1 Historical Perspective	 1
 2 The Albumin Molecule: Its Structure and Chemical Properties	
I. Primary Structure and Composition	9
A. Amino Acid Sequence and Disulfide Bonding Patterns	9
B. Composition	14
C. Features	17
D. Fragments	19
II. Tertiary Structure and Physical Chemical Behavior.....	24
A. Configuration	24
B. Physical Chemistry	39
C. Changes in Configuration	54
 3 Ligand Binding by Albumin	
I. Anionic and Neutral Ligands	79
A. Long-Chain Fatty Acids	79
B. Bilirubin	95
C. Site-I Ligands	102
D. Site-II Ligands	109
E. Miscellaneous Anionic and Neutral Ligands	116
II. Cationic Ligands	121
A. Copper(II) and Nickel(II)	121

B. Calcium and Magnesium	124
C. Other Metals and Cationic Drugs	126
III. Antibodies; Immunochemistry of Albumin	127
A. Number and Distribution of Determinants	128
B. Sequences and Chemical Groups Involved	129
C. Albumin Immunology <i>in Vivo</i>	131

4 Genetics: The Albumin Gene

I. Gene Structure	133
A. Albumin Gene Sequence	134
B. Codon Usage	141
C. Polymorphisms	142
II. Close Relatives: The Albumin Superfamily	143
A. α -Fetoprotein	143
B. α -Albumin	149
C. Vitamin D-Binding Protein	150
III. Evolution: Origins in the Past	153
A. Identification	154
B. Possible Origins	167
IV. Mutant Forms	170
A. Classification	170
B. Molecular Location of Mutations	171
C. Distribution in Populations	173
D. Effects of Mutations on Albumin Molecule	178
E. Genetics of Analbuminemia	181

5 Metabolism: Albumin in the Body

I. Biosynthesis	188
A. Systems Used to Study Albumin Biosynthesis	189
B. Expression (Transcription)	192
C. Translation	206
D. Secretion: Proalbumin and Its Possible Roles	213
E. Rate of Biosynthesis	223
II. Distribution, Functions, and Fate in the Body	228
A. Distribution	228
B. Functions	234
C. Survival in Analbuminemia	240
D. Changes to Albumin in Circulation	243
III. Degradation: Role in Nutrition	245
A. Rate of Degradation	245

B. Site(s) of Degradation	247
C. Mechanism of Degradation	248
D. Fate of Degradation Products	249
 6 Clinical Aspects: Albumin in Medicine	
I. Assays	251
A. Methods	251
B. Standardization	255
II. Pathology: Changes in Disease	256
A. Albumin Concentration and Its Significance	256
B. Relation of Albumin to Some Metabolic Diseases	260
III. Parenteral Uses	278
A. Circulatory Support	279
B. Digestive Support	280
C. Removal of Toxins	281
D. Imaging	281
E. Drug Delivery	283
F. Coating Surfaces	284
 7 Practical Aspects: Albumin in the Laboratory	
I. Methods of Preparation	285
A. Laboratory Purification	286
B. Commercial Purification	291
C. Recombinant Production	296
II. Further Purification: Albumin Heterogeneity	298
A. Associated Substances	298
B. Forms of Albumin Observed	301
III. Albumin Products and Their <i>in Vitro</i> Applications	305
A. Products	305
B. <i>In Vitro</i> Applications	308
IV. Tested Procedures for Use in Laboratory	312
A. Preparing Solutions of Albumin	313
B. Characterizing Albumin Preparations	313
C. Isolating Albumin from Serum	315
D. Modifying Albumin	316
 Bibliography	
Index	415

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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.

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.

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

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-

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

HIV	Human immunodeficiency virus	NIDDM	Non-insulin-dependent (Type-II) diabetes mellitus
HMA	Human mercaptalbumin	NMR	Nuclear magnetic resonance
HPLC	High-performance liquid chromatography	NSA	Normal serum albumin (commercial fraction V for IV use)
HSA	Human serum albumin	ODMR	Optically detected magnetic resonance
IDDM	Insulin-dependent (Type-I) diabetes mellitus	ORD	Optical rotatory dispersion
Ig	Immunoglobulin	PCR	Polymerase chain reaction
IL	Interleukin	PE	Proximal element (genetics)
IR	Infrared	PEG	Polyethylene glycol
kb	Kilobase	RER	Rough-surfaced endoplasmic reticulum
kDa	Kilodaltons	RFLP	Restriction-fragment length polymorphism
L	Liter	RSA	Rat serum albumin
LCFA	Long-chain fatty acids, C ₁₆ –C ₂₀	s	Second
<i>M</i>	Moles/liter	SE	Sulfoethyl
MCFA	Medium-chain fatty acids, C ₆ –C ₁₄	T3	Triiodothyronine
M/M	Mole/mole	T4	Thyroxine
MMADS	Monoacetyldiaminophenyl sulfone (bilirubin analog)	TCA	Trichloroacetic acid
mRNA	Messenger ribonucleic acid	TNF	Tumor necrosis factor
My	Million years (evolution)	tRNA	Transfer RNA
NAR	Nagase analbuminemic rat	UV	Ultraviolet
NASA	National Aeronautic and Space Agency	°	Degree Celsius

2

The Albumin Molecule: Its Structure and Chemical Properties

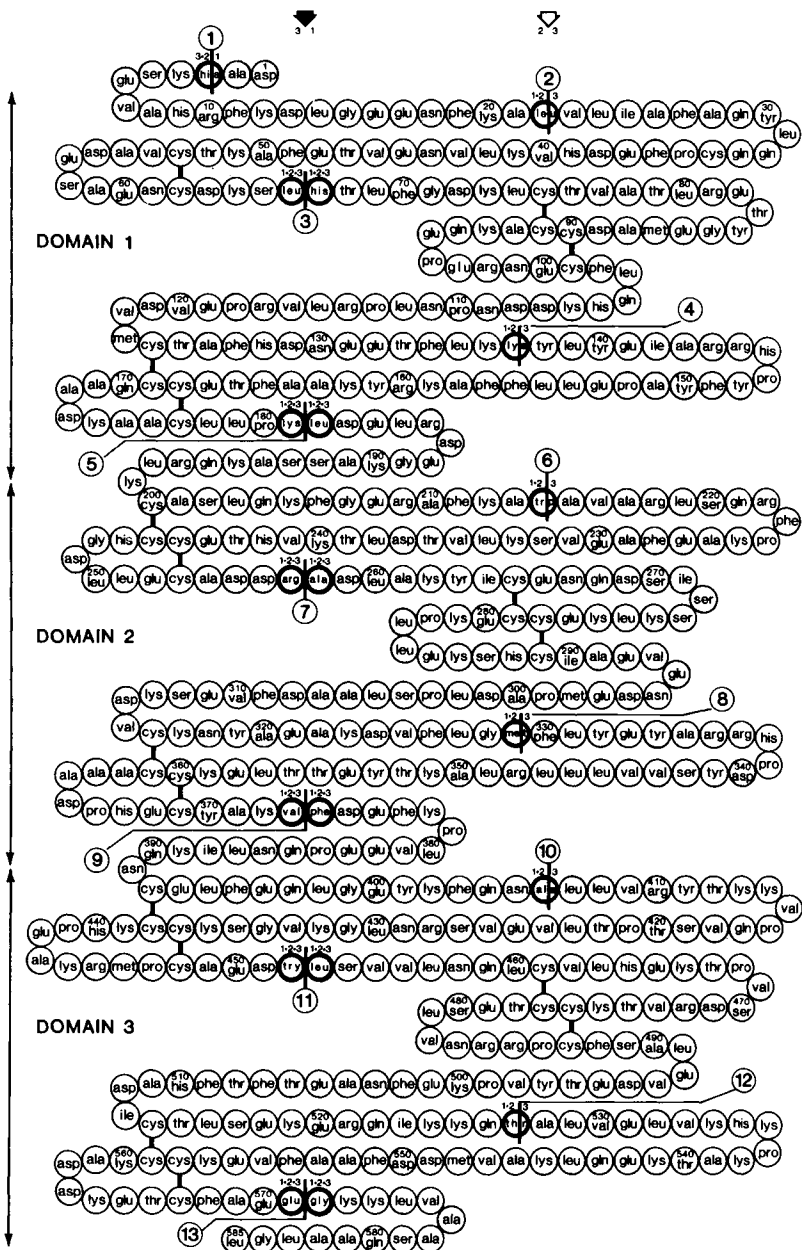
This chapter concentrates on structural information about human and bovine albumins: human albumin for its obvious importance in clinical, metabolic, and genetic studies, and bovine albumin for its role as a model protein and its many *in vitro* applications. Amino acid composition and sequence data 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



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 Rovey, 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

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 Dugaiczky *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 Dugaiczky 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

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-

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,B5, 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.

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	66,411.17	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	

^aFrom Fig. 2-1.^bFrom Fig. 2-2.^cFrom Sargent et al. (1981b).^dFrom 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

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).

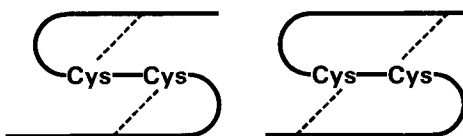


Fig. 2-3. The two possible conformations of disulfide bonds at a Cys-Cys pair, modified from Foster (1960).

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 II 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

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 ($0.033\text{ }M$, 105°) releases principally aspartic acid from albumin (Grannis, 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

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 <i>Streptococcus</i> protein G
9	P-Phe	495-585 ³²	506-583 ^{2, 13, 33}	Contains no Tyr/Phe/Trp

^aRat 1-24 (from Ref. 1), 1-308 (from Ref. 10).

^bKey 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).

(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.

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-

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 $1\text{--}2\ \mu\text{M}^{-1}$ (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.

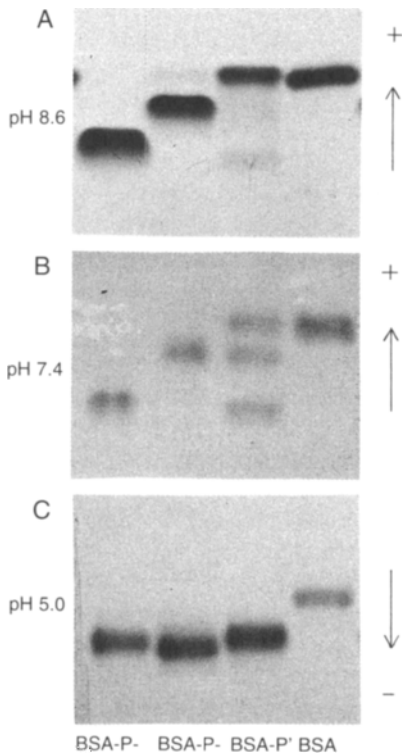


Fig. 2-4. Electrophoresis on cellulose acetate demonstrating the association of two large fragments of BSA to form a molecule similar to BSA. Left to right: BSA 308–583, BSA 1–307, equimolar mixture of the two fragments, intact BSA. (A) pH 8.6; (B) pH 7.4; (C) pH 5.0. The anode is at the top. The fragments 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.

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 ultracentrifugal 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

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 $D_T (\times 10^{-13} \text{ m}^2 \text{ s}^{-1})$	628	—
Partial specific volume V_{20}	0.7336	0.7336
Intrinsic viscosity, η	0.0426	0.0417
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, $\Gamma/2 = 0.15$ $10^5 \text{ cm}^2 \text{ v}^{-1} \text{ s}^{-1}$	-5.9 ¹⁹	—
Net charge per molecule pH 7.4	-192 ⁰	—
Calculated from Table 2-1	-15	-17
Refractive index increment (578 nm), $\times 10^{-3}$	1.892 ¹	1.909
Optical absorbance, 279 nm, 1 g L ⁻¹	0.531 ²²	0.667 ²³
Mean residue rotation $[m']_{233}$	8590 ²⁴	8443 ²⁵
Mean residue ellipticity (theta)		
$[\theta]_{209} \text{ nm}$	1726	21.127
$[\theta]_{222} \text{ nm}$	16	20.1
Estimated α helix, %	67 ¹⁴	68 ²⁸
β form, %	10	17

^aKey to references (superscript numbers): (1) Charlwood (1961), (2) Squire *et al.* (19688), (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) Longworth 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).

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 $40 \times 140 \text{ \AA}$ 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 $41.6 \times 140.9 \text{ \AA}$. Studies of the frequency dispersion of the dielectric constant of human (Scheider *et al.*, 1976) and bovine (Rosseneu-Motreff *et al.*, 1973) albumins (Section II,B,2) predicted a 3:1 axial ratio. For HSA Oncley *et al.* (1946) predicted dimensions of $38 \times 150 \text{ \AA}$ 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

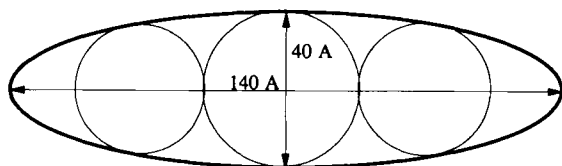


Fig. 2-5. Model of the HSA molecule derived from hydrodynamic studies.

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 $41 \pm 7 \times 134 \pm 22$ 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

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 Å³. 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 *P*2₁2₁2, 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-

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 $P4_212$, 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 polyaniline 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 $\beta = 101.9^\circ$. They were much drier, with solvent content only $\approx 33\%$. 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).

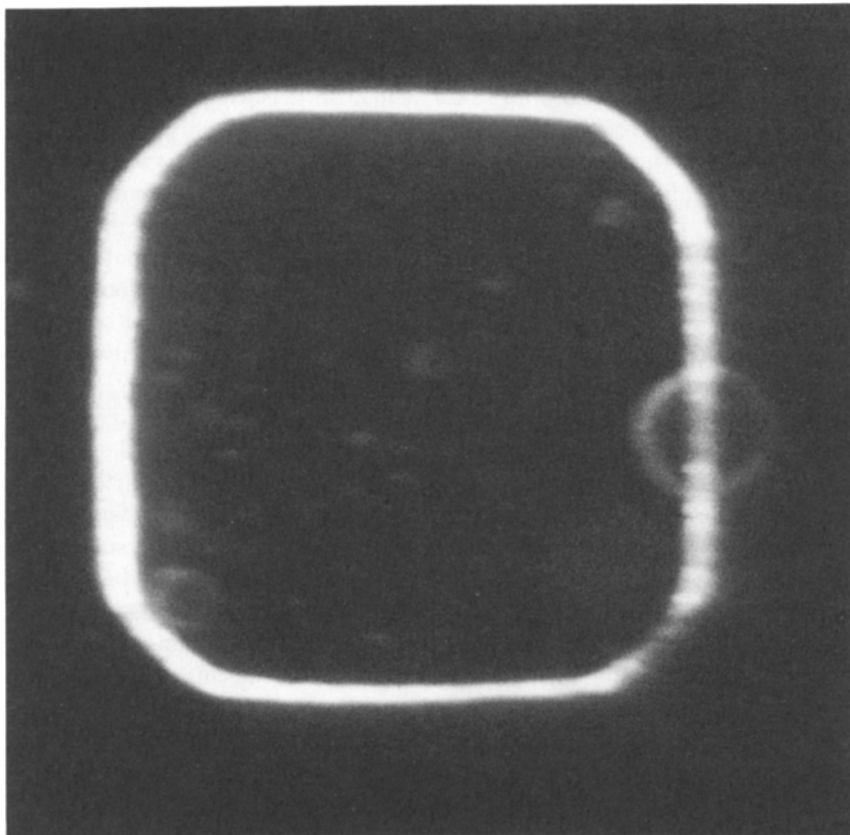


Fig. 2-6. Tetragonal crystal of HSA. Photograph by D.C. Carter of the National Aeronautic and Space Agency, Huntsville, Alabama.

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 Å³ (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 Trp²¹⁴-Try⁴¹¹ (19 Å), Trp²¹⁴-Cys³⁴ (33 Å), and Tyr⁴¹¹-Cys³⁴ (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

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

Fluorescent energy transfer predicted the Trp²¹⁴-Tyr⁴¹¹ 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 diagrammed 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 (color-plate) 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

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 heart-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.]

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 $\pm 80^\circ$.

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.

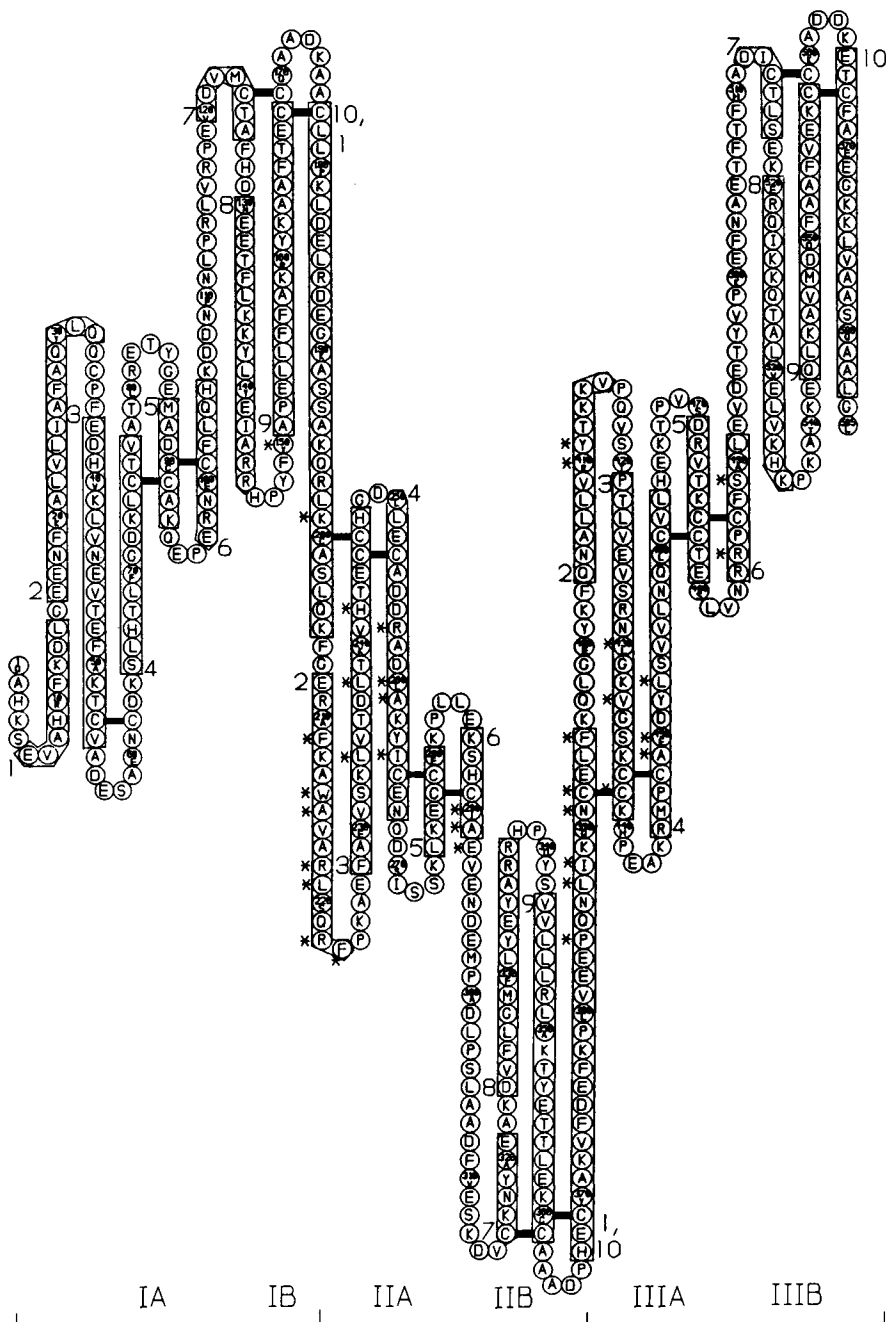
Nearly all of the mutant sites identified so far lie on the surface of the albumin structure (considered in more detail in Chapter 4, Section IV). Similarly, antigenic sites, or epitopes, can be found on the exterior (Chapter 3, Section III). CySH-34, the sole thiol group, and some other active residues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



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.

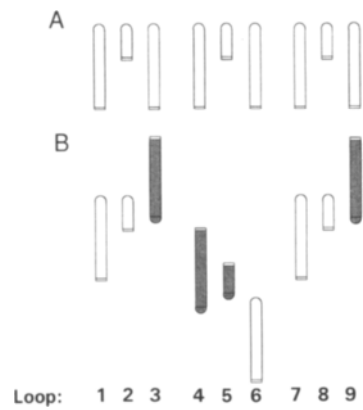


Fig. 2-10. Relationship of the X-ray heart-shaped structure (B) to the linear model (A). Loops in an inverted orientation are filled. Note that loops 3, 4, 5, and 9 are inverted and the long loops 3, 6, and 9 are displaced from the linear formation.

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

Name	Domain I		Domain II		Domain III	
	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

^aFrom Carter and Ho (1994).

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		

^aFrom Carter and Ho (1944)

^bMore remote but still form important sides of the binding site.

^cStill 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

ultrasonic spectroscopy and found that expansion and helix-coil transitions relate to pressure wave absorptions at 200 kHz, corresponding to times of 5 μ sec 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 Å² compared to only 10 Å² 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.

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, $\epsilon_{280 \text{ nm}}$, 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 tryptophan compared to two for BSA. The absorptive peaks for HSA and BSA are not exactly at 280 nm, the

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 $A_{280\text{ nm}}$ for albumins from their composition, including a contribution from S–S bonds ($\epsilon_{280\text{ nm}} = 134$), 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

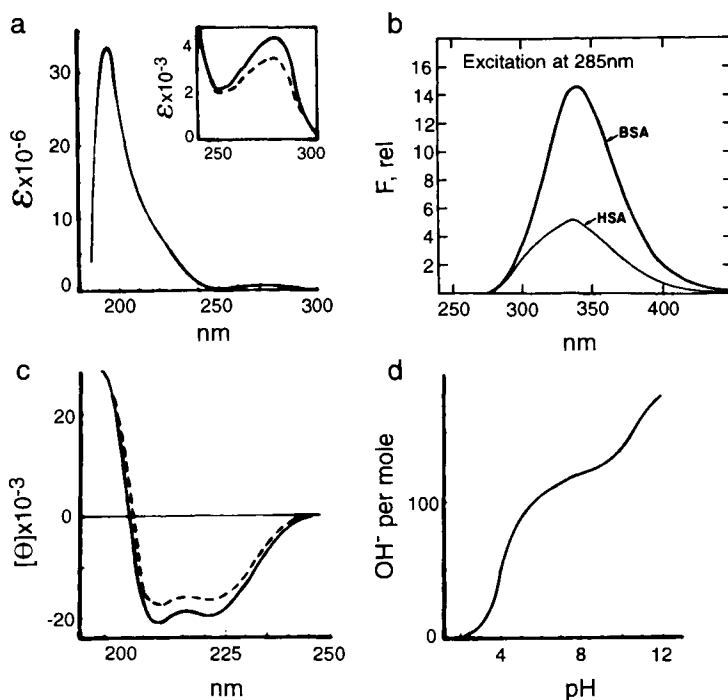


Fig. 2-12. Some physical chemical properties of albumins. (a) Molar absorptivity; (b) fluorescence emission (relative); (c) mean residue ellipticity by CD; (d) acid–base titration curve. Solid lines, BSA; dashed lines, HSA. The titration curves of HSA and BSA do not differ significantly. Modified from 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.

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. $A_{1\text{ g/L}}$ 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.

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 $\lambda_{\max}$ 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

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'

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

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:

$$\text{Cl}^-/\text{albumin (mol/mol)} = 13.5 + 5.6 \log [\text{Cl}^-] \text{ (in mol/kg H}_2\text{O)}. \quad (1)$$

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

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 mM) 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 $p\text{O}_2$, 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 pK 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.

Table 2-6, modified from Foster with current analytical data, lists the numbers of potentially ionizable groups and their average intrinsic pK 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 $\rightarrow$ F isomerization (Section II,C,1). About half of the carboxyls are considered to ionize with an intrinsic pK 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 pK 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

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 ^c	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

^aFrom titration data of Tanford (1950) and Foster (1960), calculated to 66,500 Da.

^bFrom Figge *et al.* (1992).

^cFrom Sadler and Tucker (1992).

^dFrom Lewis *et al.* (1980).

^eComposition 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°

with rotary diffusion constants of 4×10^6 and $1 \times 10^6 \text{ s}^{-1}$, 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.

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 salicylate 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 glycosylated 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 tyrosine 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 guanidinyll 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).

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 methionyl 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 μ M of homocysteine is also found (Fiskerstrand *et al.*, 1993), corresponding to 2% of the bound cysteine.

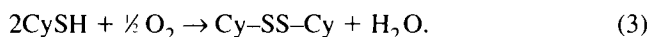
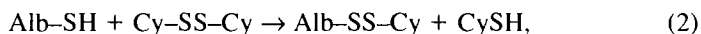
A preparation of albumin containing no mixed disulfide, in which all of Cys-34 is in the SH or mercaptan form, is termed *mercaptalbumin*, often abbreviated HMA or BMA for the human or bovine species. HMA was first isolated by Hughes of the laboratory of E. J. Cohn, after crystallizing HSA dimers formed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

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 ($\approx 0.05 M$) 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.



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.

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 (Hermanson *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 ($\cdot\text{OH}$), superoxide (O_2^-), and hydrodioxy ($\text{HO}_2\cdot$) 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 $\approx$ 9. The albumin thiol, on the other hand, appears to be considerably more acidic, with a *pK* 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 *pK* of 8.9, normally its apparent *pK* 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 sub-domain 1A (Fig. 2-11). Tyr-30 lies $\approx$ 10.3 Å away, buried in helix h2, and Tyr-84, partially exposed in the short stretch between helices h4 and h5, is only

≈ 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

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 Å². 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

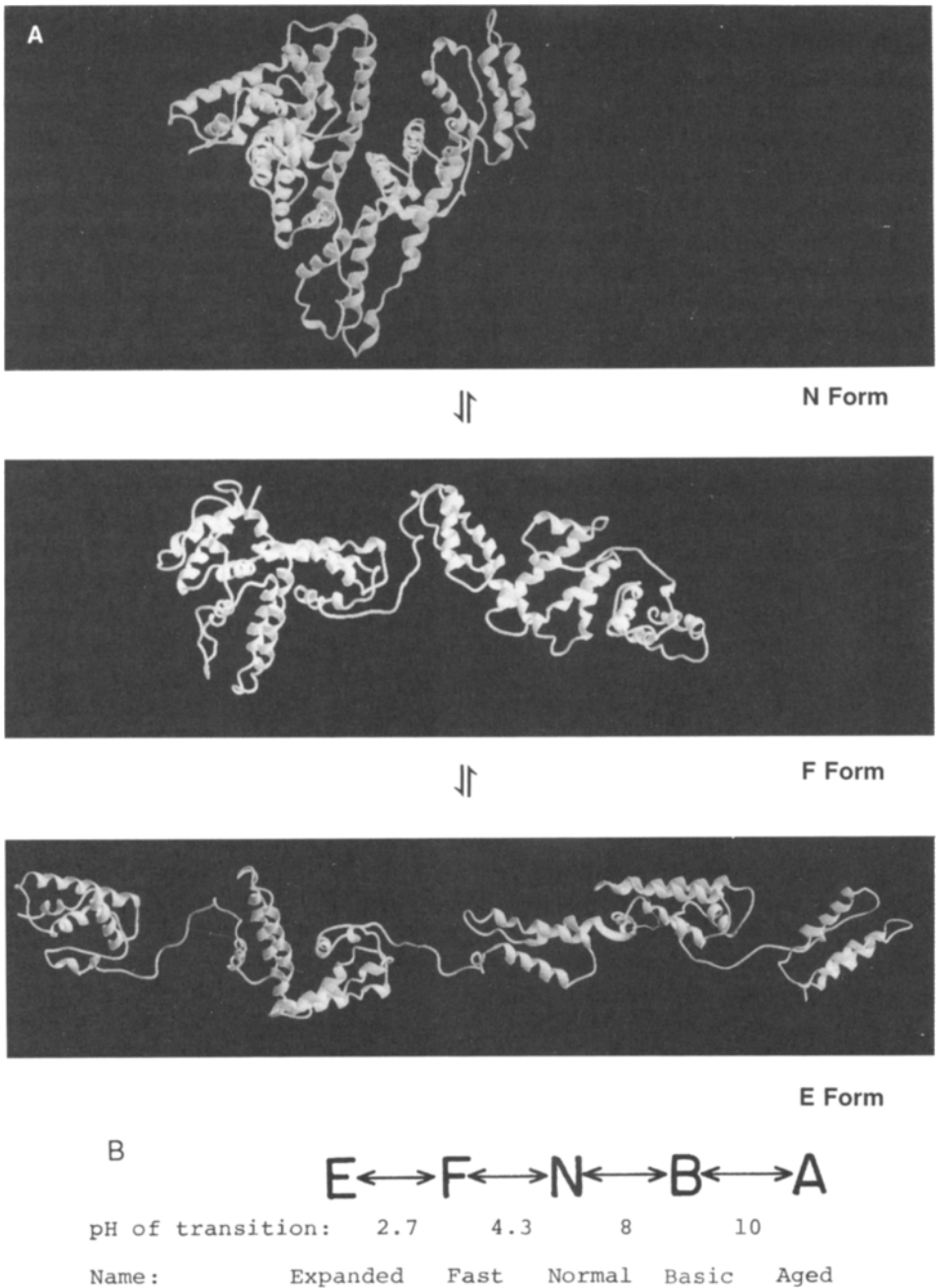


Fig. 2-14. (A) Proposed configurations of **F** and **E** isomeric forms of HSA. Reproduced from Carter and Ho (1994) by permission of Academic Press. (B) Interrelation of five recognized forms. Reproduced from Peters (1985) with permission of Academic Press.

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 ^1H 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, $\text{N} \rightarrow \text{F1} \rightarrow \text{F}$. 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 Gln³⁹³-Phe³⁹⁴ (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, Asp³⁰⁷-Phe³⁰⁸, 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

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 $\rightarrow$ 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.

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 pK of ionizable groups, in this case imidazoles 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 rigidity, 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 Å² only to 47,000 Å² (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** $\rightarrow$ **B** transition, 0.002 M Ca²⁺ 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

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.

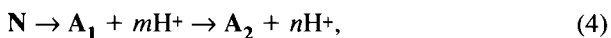
Effects on particular residues support this concept. ¹H NMR chemical shifts showed several histidine C-2 atoms to have unusually high pK values, being protonated above pH 7 in the **N** form; five of these were seen in domain I and were affected by calcium, causing Bos *et al.* (1989b) to assign them 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 ¹⁹F 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

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),



with isoelectric points of 5.39 and 5.45, respectively, for the **A**₁ and **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.

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, D₂O, 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 mM CaCl₂ lowers the equilibrium value to about 10%. ClO₄[−] 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), C₆ and C₈, impede the formation, and C₁₀ and C₁₂ 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. Because 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 through 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 concerning the behavior of albumin under various denaturing conditions owing to its role as a model protein. Some salient fea-

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):

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

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 $\approx$ 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

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 sub-zero 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.

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 $\approx$ 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.

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

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 >10⁶ 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 mM 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

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 concentration (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 Kilibanov, 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 $\mu\text{g}/\text{cm}^2$ (Mura-Galelli *et al.*, 1991). As far back as 1947, Bateman found a figure of 0.13 $\mu\text{g}/\text{cm}^2$ during the use of films as an assay procedure for plasma proteins, and Bull (1947) in his review reported a figure of 0.135 $\mu\text{g}/\text{cm}^2$.

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 $\mu\text{g}/\text{cm}^2$, the *calculated* area of an albumin molecule on a surface is 7070 $\text{\AA}^2$. This implies that a triangular albumin molecule with equilateral 80- $\text{\AA}$ sides and a 29- $\text{\AA}$ average thickness has become flattened to 127- $\text{\AA}$ sides and an 11.6- $\text{\AA}$ average thickness, a flattening of 2.5-fold.

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 Chatteraj, 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

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 mM 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

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 mM 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 (Lamiet and Plückthun, 1989). The driving forces affecting the unfolded protein are strongly influenced by the surrounding solvent medium.

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 *intermolecular* bonding. Conditions found effective for refolding are 1–2 μM albumin, pH 8.0, 0.1 *M* Tris-Cl buffer, 1 *mM* EDTA, 1 *mM* reduced glutathione, and 0.1 *mM* 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 μM about half of the albumin polymerizes. At 9 μM oligomers form rapidly but convert slowly to monomer (Wichman *et al.*, 1977). Concentrations as low as 0.5 μM ($\approx 30 \mu\text{g/mL}$) 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

stimulated at 280 nm (Andersson, 1969). Binding of fluorophores 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 conditions were pH 10, 1 mM 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

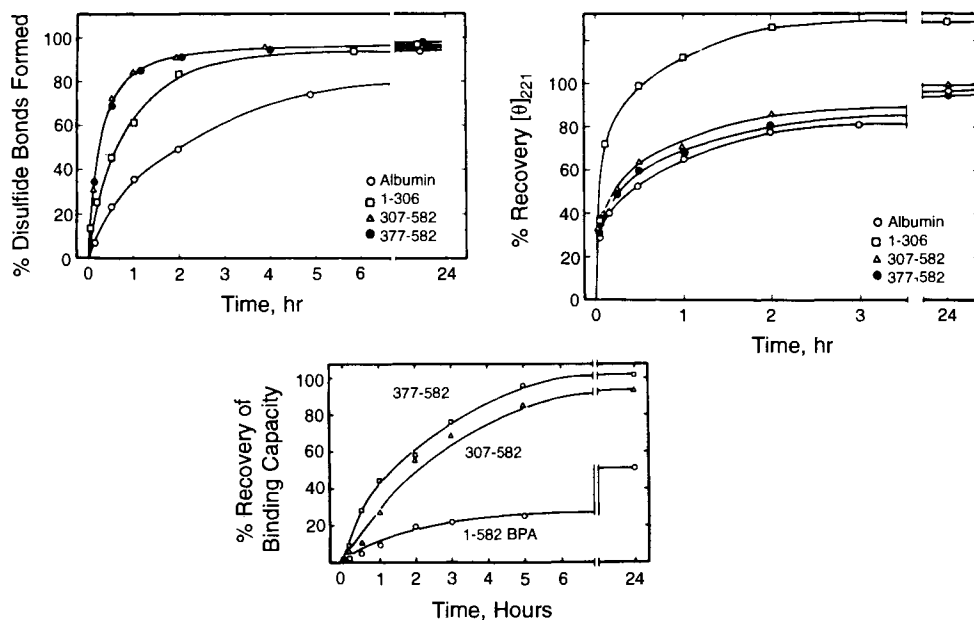


Fig. 2-15. Refolding of reduced BSA and some of its fragments. Upper left: Appearance of protein S-S bonds with time. Upper right: Return of mean residue ellipticity. Bottom: Regeneration of palmitate-binding capacity. Residue numbers 306 and higher should be increased by 1. From Johansen *et al.* (1981)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

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,

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.

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:

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	$(1-69) \times 10^7$	1	Richieri <i>et al.</i> (1993)
Eicosanoids (PGE ₁)	7×10^4	2	Unger (1972)
Bile acids ^b	$(3-200) \times 10^3$	3	Roda <i>et al.</i> (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 <i>et al.</i> (1977)
Bilirubin	9.5×10^7	1	Brodersen (1982)
Hematin	1.1×10^8	1	Adams and Berman (1980)
L-Thyroxine	1.6×10^6	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 <i>et al.</i> (1986)
1,25-(OH) ₂ -Vitamin D ₃	5×10^4	1	Bikle <i>et al.</i> (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 <i>et al.</i> (1993)
Zinc(II)	3.4×10^7	1	Masuoka <i>et al.</i> (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	

^aSee Table 3-2.
^bSee Table 3-4.
^cWith 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.

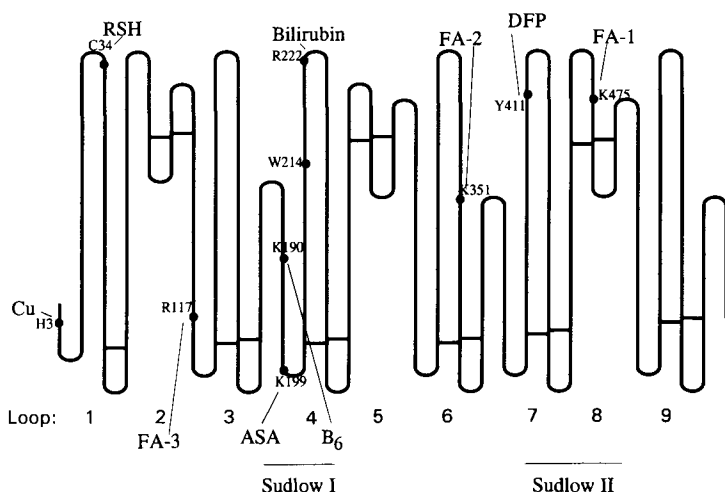


Fig. 3-1. Schematic of binding site locations on HSA. FA, Long-chain fatty acids; ASA, acetylsalicylate; B₆, pyridoxal 5'-phosphate; RSH, mixed disulfides; DFP, diisopropyl-fluorophosphate.

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.

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 1mM, 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 nM; aggregation of like molecules into micelles brings the unbound fatty acid concentration to about 10^{-4} mM (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 mM in plasma, and that of albumin is about

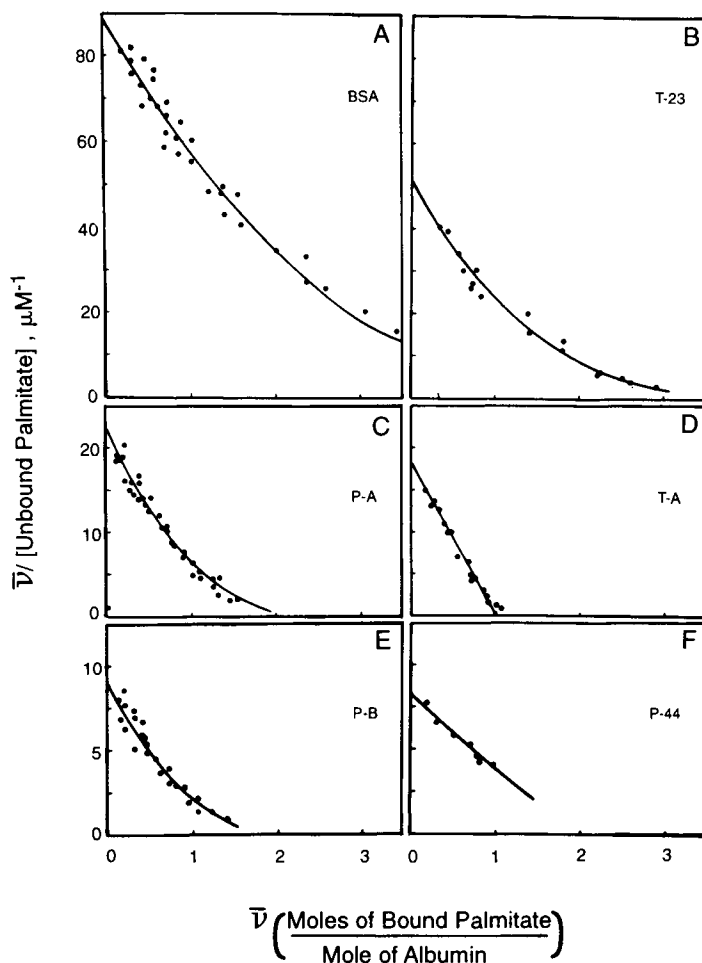


Fig. 3-2. Scatchard plots of binding of palmitate to (A) BSA and five of its fragments (see Table 2-2). Curve D (T-A) is its isolated domain III. Solid lines represent the binding curve calculated from K_A values of 34, 8.1, and $3.0 \mu\text{M}^{-1}$ for the first three sites of BSA and 18 and $<0.2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

0.6 mM, 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).

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 Lys³⁷⁶-Pro³⁷⁷ 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 guanidyl groups has been shown to interfere with fatty acid binding (Jonas and Weber, 1971).

A subsequent NMR study using bovine albumin fragments with ¹³COOH-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 ¹H 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 LCFAs 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

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 $\beta\beta$ 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.

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

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 nM for stearate, palmitate, and oleate, rising to about 20 nM for linoleate and arachidonate and 50 nM 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 $[HSA] \approx 6 \mu M$, 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 ($\approx 600 \mu M$).

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.

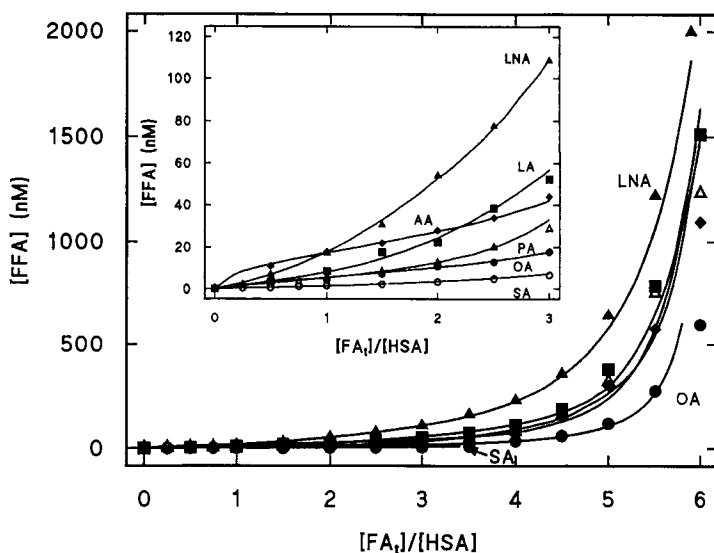


Fig. 3-3. Binding isotherm for long-chain fatty acids. SA, Stearic acid; OA, oleic acid; PA, palmitic acid; LA, linoleic acid; LNA, linolenic acid; AA, arachidonic acid; FFA, free fatty acid. Reprinted with permission from Richieri *et al.* (1993). Copyright 1993 American Chemical Society.

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

Compound	HSA		BSA	
	K_A ($10^7 \times M^{-1}$)	<i>n</i>	K_A ($10^7 \times M^{-1}$)	<i>n</i>
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	—

^aFrom Richieri *et al.* (1993).

^bFrom Klopfenstein (1969).

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 $^{13}\text{COOH}$ -stearate showed five or more binding resonances, and implied that the ligand is distributed 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).

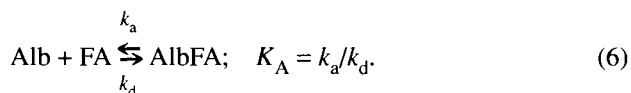
These oleates are constantly exchanging among sites on albumin molecules via the free state. As noted in Chapter 2 (Section II,C,2,c), when fat-free albumin molecules, which are more susceptible to heat denaturation, are removed by heat coagulation, the remaining molecules accumulate more and more oleate 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

^aFrom Spector and Fletcher (1978).

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



Svenson *et al.* (1974) estimated the dissociation rate constant from the speed of uptake of palmitate from soluble HSA to immobilized HSA as 0.04s^{-1} at 23° . Speeds of uptake must be much higher to yield K_A values $> 10^{-8} \text{ M}^{-1}$. 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 ($< 1 \text{ ms}$) 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 $T\Delta S = -8 \text{ kcal mol}^{-1}$. 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: $\Delta H = -10$, $T\Delta S$ 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, $\text{C}_8\text{--C}_{14}$, 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, $\text{C}_{16}\text{--C}_{20}$, which are

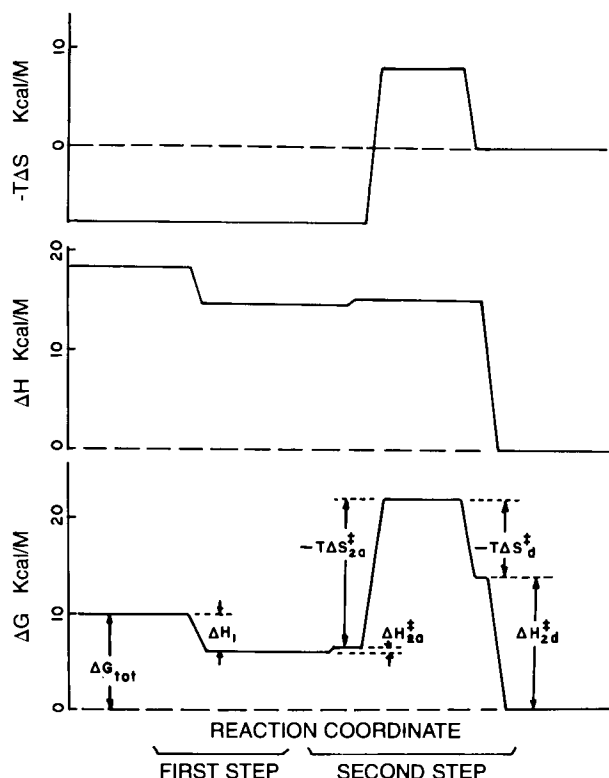


Fig. 3-4. Thermodynamics of long-chain fatty acid binding. Reproduced from Scheider (1979).

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 LCFA 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 $143 \times 34 \text{ \AA}$ to $128 \times 41 \text{ \AA}$.

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

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 $\bar{\nu} = 0\text{--}2$ (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

lysophosphatidylcholine binding (Rosseneu-Motreff *et al.*, 1974), so lyso-lecithins 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

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 Wynaalda, 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_4 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).

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 $1 \mu\text{g/L}$, 95% was bound by transcortin but at higher concentrations the binding became nonlinear and fell to 80% at $1.8 \mu\text{g/L}$. 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 $4.6 \text{ mg}/100\text{mL}$ 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

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 $5 \times 10^4 M^{-1}$, respectively, for the mono and 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 decreases 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 $25 \times 10^4 M^{-1}$ 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

TABLE 3-4
Binding of Bile Acids to Albumin^a

Compound	Association constant, K_A ($10^4 \times M^{-1}$)	<i>n</i>
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

^aFrom 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

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 IX α 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 IX α 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 nM 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

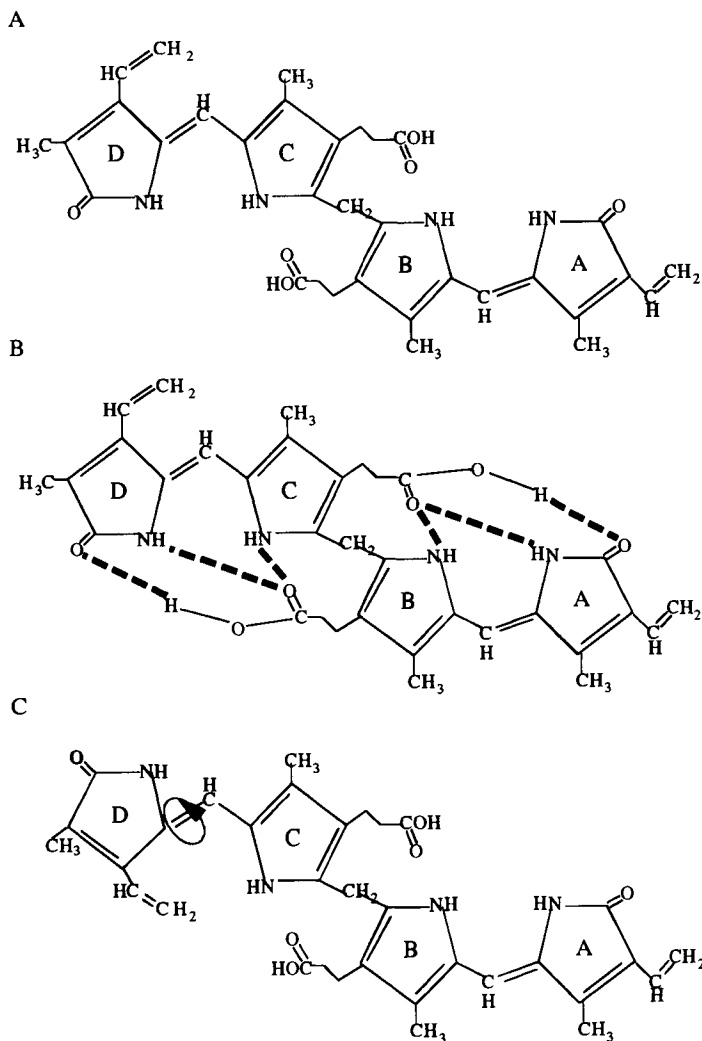


Fig. 3-5. Structure of bilirubin IX α molecule: (A) ZZ form in planar presentation; (B) ZZ, folded, with hydrogen bonds; (C) ZZ $\rightarrow$ ZE conversion. Based on the work of Brodersen (1980).

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

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 $\frac{1}{10}$ 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,

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 IX α 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 Δ Z4, Δ Z15, 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,E15 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

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 circulating 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 demonstrates 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

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.

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** $\rightarrow$ **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 4.7×10^6 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 -CHOHCH₃ side chains of either -CH=CH₂ or -H alone raises the K_A markedly to 280×10^6 or 450×10^6 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.

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** $\rightarrow$ **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).

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 <i>et al.</i> (1986)
Tolbutamide	4×10^4	1.4	1	Vallner (1977)
Furosemide	2.6×10^4	1.6	3	Viani <i>et al.</i> (1991b)
Phenytoin	6×10^3	6	9	Schoenemann <i>et al.</i> (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 <i>et al.</i> (1973)
Phenol red	2.8×10^4	1	—	Rodkey (1961)
Bromocresol green	7×10^5	3	—	Rodkey (1964)
Bromphenol blue	1.5×10^6	3	—	Bjerrum (1968)
Iophenoxate	8×10^7	—	—	Mudge <i>et al.</i> (1978)
Sulfobromophthalein	1.7×10^7	—	—	Baker and Bradley (1966)
Methyl orange	2.2×10^3	—	—	Klotz <i>et al.</i> (1946)
Methyl red	2.2×10^5	—	—	Burkhard <i>et al.</i> (1961)
Evans blue	4.0×10^5	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)

^aSome data from Wandell (1983).

^bSee also tryptophan and thyroxine (Section III,A,4) as Site-II ligands.

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.*,

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.*,

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 ^1H 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 ^1H NMR (Honma *et al.*, 1991), and the appearance of the acetyl group of aspirin as

an acetamide confirmed by ^{13}C NMR (Gerig *et al.*, 1981). Lys-199 was found to be well exposed to the solvent, and its $\epsilon\text{-NH}_2$ 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 $\epsilon\text{-NH}_2$ 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 $k_c = \sim 0.3\text{s}^{-1}$, 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

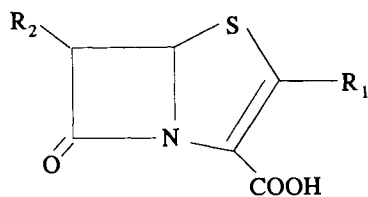


Fig. 3-6. Structure of penem group.

allergenes (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-I 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

attributed to conformational changes. An 186 Arg $\rightarrow$ 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 C₆-C₁₀ 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.

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, iopodate, 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 ¹H 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

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 nM 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, respectively, 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.

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 ($\approx$ 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 $\rightarrow$ 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, C₆–C₁₄, 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 C₁₀ 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 mM chloride being about one-third that in phosphate buffer (Honoré and Brodersen, 1988).

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) (Scatchard 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 (Sjödén *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 $3.8 \times 10^5\text{ M}^{-1}$ (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.

Microcalorimetry yields a ΔH of $-5.7 \text{ kcal mol}^{-1}$, with a strong positive effect of pH as in the **N** $\rightarrow$ **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 $10^3 \text{ M}^{-1} \text{ s}^{-1}$, 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).

An accelerated decomposition of 4-hydroxycyclophosphamide by albumin to yield phosphoramidate mustard is much slower, $k_{\text{cat}} = 285 \text{ M}^{-1} \text{ min}^{-1}$ (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 $8 \times 16 \text{ Å}$ (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

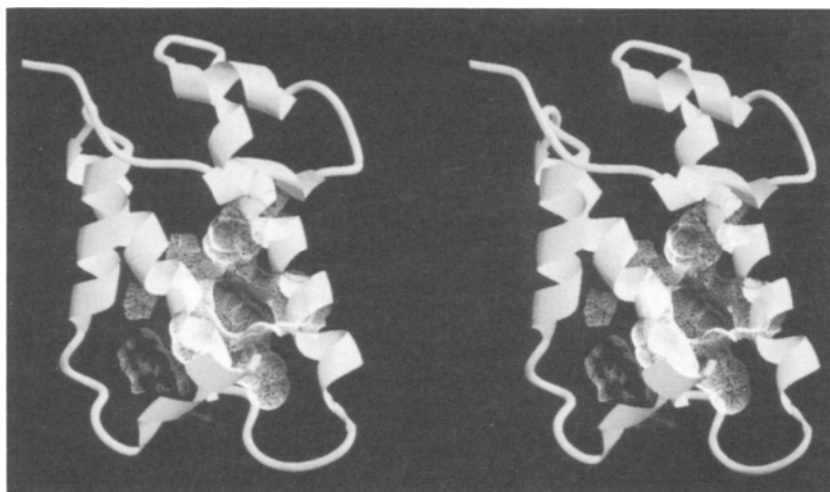


Fig. 3-7. Dotted-surface ribbon diagram of the major binding pocket inside subdomain IIIA (Sudlow Site II), with ligand. Reproduced with permission of the authors and Academic Press, from Carter and Ho (1994).

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 ($\bar{\nu} = 1\text{--}3$) 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 ($\approx 15\%$) 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.

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*-nitroso proteins, which extends its half-life to the order of hours. Of the total NO in plasma, $\approx 7 \mu\text{M}$, 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 $K_A = 3 \times 10^3 \text{ M}^{-1}$ (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).

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-I 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 B₆ 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 B₁₂ 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

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). Isoflurane ($\text{CHF}_2\text{OCHClCF}_3$) and halothane were shown by ^{19}F NMR to bind to BSA in a weak ($K_A \approx 800 \text{ M}^{-1}$) but specific manner (Dubois *et al.*, 1993). Halothane increased the binding of warfarin, and trifluoroacetate decreased both warfarin and phenytoin binding. Anazoline 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 glycosylated form.

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

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 (Sjöbring *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 (Lämmle *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 repetitive sequences and are situated toward 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

the C-terminal domain, particularly loops 6–8 and perhaps loop 8 alone (see Table 2-2) (Widebäck, 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*-methanamide, and with near-full affinity by the generic analog, Gly-Gly-His-*N*-methanamide, 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 -NH₂ nitrogen, the nitrogen atoms of the first two peptide bonds, and the 3-nitrogen of the histidine imidazole ring (Fig. 3-8). ¹H 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).

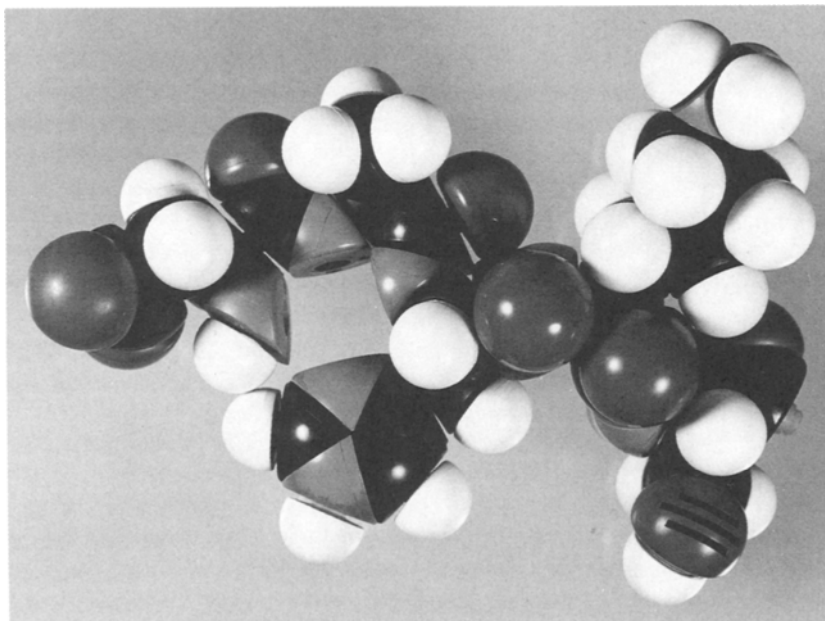


Fig. 3-8. Molecular model of structure of Cu(II) binding site of BSA. Reproduced from Peters and Blumenstock (1967) by permission of the American Society for Biochemistry and Molecular Biology.

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 $\lambda_{\max} = 525 \text{ nm}$ and $\epsilon_{\max} = 101 \text{ L M}^{-1} \text{ cm}^{-1}$ (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

strong alkali ($\lambda_{\max} = 540$ 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 $\beta\text{-COO}^-$ 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 $\text{mol}^{-1} \text{deg}^{-1}$, 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, $K_A = 4 \times 10^9 \text{ M}^{-1}$, 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, $\lambda_{\max} = 420$ nm, $\epsilon_{\max} = 137 \text{ L M}^{-1} \text{ cm}^{-1}$ (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 μM , so that the site on albumin is only about 0.3% occupied. The plasma concentration of nickel is $<0.03 \mu\text{M}$; 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 $\epsilon_{\max} = 98 \text{ L M}^{-1} \text{ cm}^{-1}$ (Lau *et al.*, 1989). It will receive copper from albumin, and presumably has a role in handling of this ion in the fetus.

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 mM 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 calcium, but unfortunately is not used routinely because the temperature, pH, and $p\text{CO}_2$ 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 mM, and bound calcium, 0.9 mM, 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

I, where ^1H 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 $\rightarrow$ 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 $14 \text{ cal mol}^{-1} \text{ deg}^{-1}$ (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** $\rightarrow$ **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 mM; 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.

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 $\approx 15 \mu\text{M}$ concentration in plasma about 65% is loosely bound and considered to be linked to albumin (Vallee and Falchuk, 1993). Thus $\bar{\nu} \approx 0.02 \text{ M/M}$ would be on albumin. Of the remainder a small amount is found with transferrin and about $2 \mu\text{M}$ 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 $\text{p}K_{\text{A}}$ 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, $\text{p}K_{\text{A}}$ 7.5, is included in Table 3-1. Cadmium binding is similar, with $\text{p}K_{\text{A}} = 5.3$ ($n = 2$). 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 -Fetoprotein binds zinc about $3\times$ 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, $\text{p}K_{\text{A}} = 12.2$, and is the major carrier. Normally total Al(III) is $<0.4 \mu\text{M}$ in plasma, but in patients on renal dialysis, when plasma levels can reach $5 \mu\text{M}$, the proportion on albumin reaches 34% (Fatemi *et al.*, 1991).

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

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 $K_A = 6.5 \times 10^3 \text{ M}^{-1}$ (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 $<0.01 \mu\text{M}$.

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 $\text{p}K_A$ 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 anti-serum 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.

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 monoclonal 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 Å². 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).

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 (Goetzel 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,

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-I, 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-Flanagan 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,

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 (Horejsi 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 idotype recognition is seen in the action of a monoclonal anti-BSA antibody, 3C-7, to suppress the T-cell response when

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.

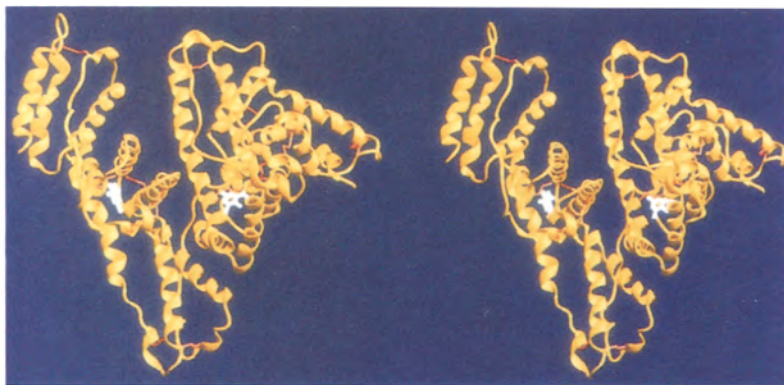


Fig. 2-7.

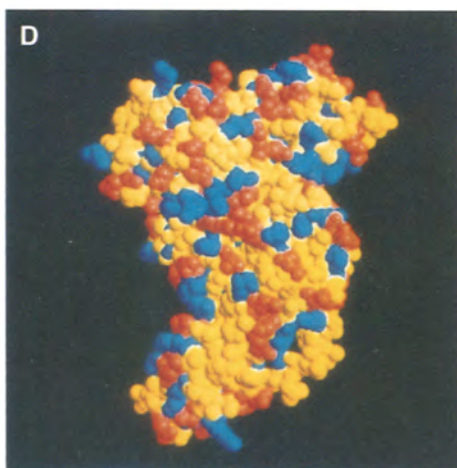
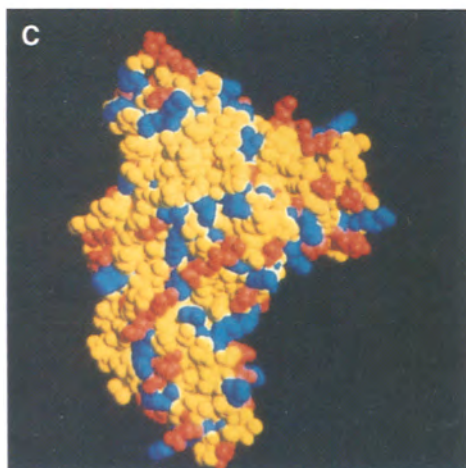
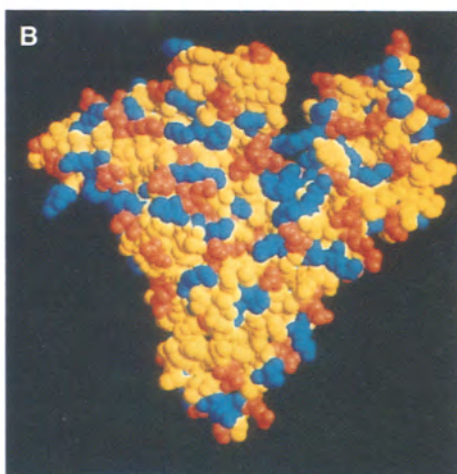
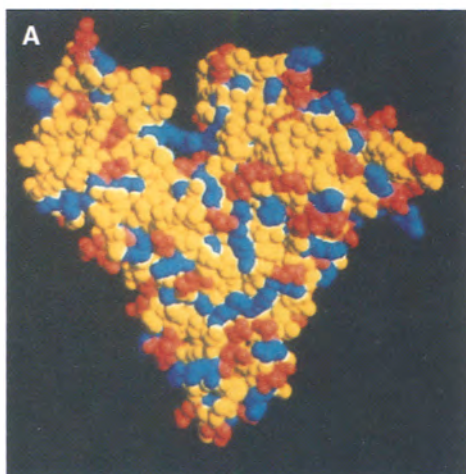


Fig. 2-8.

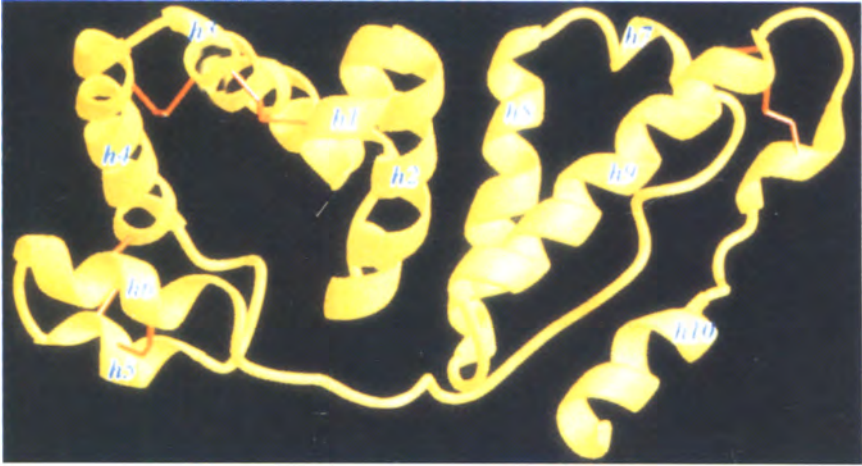


Fig. 2-11.

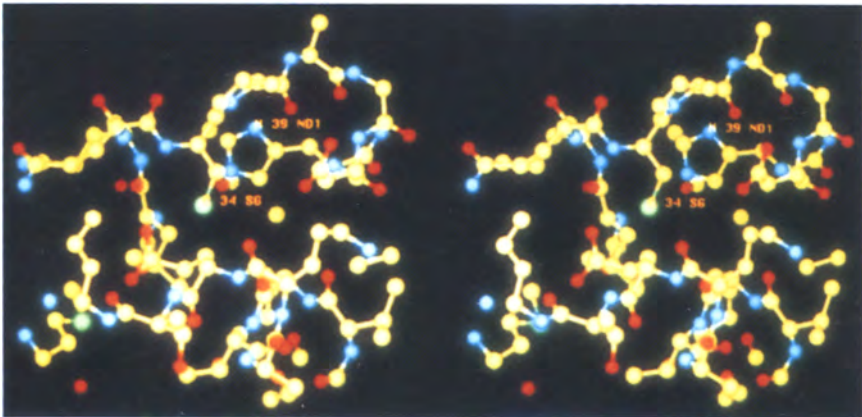


Fig. 2-13.