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Molecular Aspects of Ligand Binding to Serum Albumin

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I. Introduction

SERUM albumin is the most abundant protein in blood plasma and serves as a depot protein and transport protein for numerous endogenous and exogenous compounds. Among the endogenous substances bound with a high affinity to the protein are long-chain fatty acids and bilirubin. This interaction results in an increased solubility in plasma of both types of ligands. Furthermore, the toxicity of bilirubin is decreased in this way. Albumin is also the principal factor in contributing to the colloid osmotic pressure of the blood and has been suggested as a possible source of amino acids for various tissues (206). The physiological importance of albumin and the relative ease with which it can be isolated and purified on a large scale have resulted in a great number of binding studies. Furthermore, such investigations have been stimulated by the successful determination of the primary sequence of human [Behrens et al. (11) and Meloun et al. (168)] and bovine serum albumin [Brown (33)].

Reviews have previously appeared dealing, in relatively general terms, with binding of small molecules to albumin and other proteins (e.g. 169, 257). A number of reviews have dealt with metabolism, structure, functions, and other aspects of albumin (e.g. 205, 206). Other authors (124, 277, 110, 125) have concentrated on a discussion of the in vivo consequences of interactions between drugs and albumin under normal and pathological conditions. In the present review attention is focused on in vitro studies contributing to information on the binding process at the molecular level. Emphasis has been placed on results obtained on the human species of serum albumin. Where appropriate the literature on human serum albumin has been supplemented with that of bovine albumin, which, however, due to a different amino acid sequence, differs in certain aspects.

In the present communication the primary sequences

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of human and bovine albumins are presented accompanied by a short discussion of the spatial configuration of the proteins. Afterwards an attempt has been made to explore the possible number of binding regions necessary to explain the binding properties of albumin. It is suggested that the pronounced capability of albumin to associate noncovalently with smaller molecules and ions is due to the existence of at least six binding regions on the protein molecule. This proposal is based upon a review of the literature describing competitive ligand binding to high affinity binding sites. The review also revealed that a conclusion of competitive binding has often been drawn on a questionable basis. This problem has been commented on both theoretically, in the Appendix, and on the basis of examples reported in the literature.

Ligand binding to chemically modified albumins and to fragments of the protein is reviewed and an assignment of the above-mentioned binding regions to the currently accepted model of the secondary structure of albumin is attempted.

Finally, the capability of albumin to change conformation in the presence and absence of ligands is discussed. It is suggested that the binding regions can change conformation to some extent during the binding process.

II. Survey of Ligands Bound to Albumin

Tables 1 to 4 summarize results published on the binding of various ligands to serum albumin. It is apparent that almost all ligands are bound to a few high affinity binding sites plus a greater number of weaker sites. Especially the endogenous compounds bilirubin, long-chain fatty acids, and haemin are bound with a high affinity (table 1). In contrast, steroids and L-thyroxine, which in vivo are bound primarily to various globulins (287, 197) and prealbumin [in the case of thyroxine (197)], possess association constants 1 to 4 orders of magnitude lower. L-Tryptophan, the only amino acid bound reversibly to albumin, also exhibits a moderate binding affinity (cysteine is able to bind covalently to the SH-group of the protein). A great number of negatively charged and electrostatic neutral drugs (table 2) are bound to albumin with comparable association constants (10^4 to 10^5 M^{-1}). However, very high values have been reported for iophenoxate and iopanoate (180), which are used as cholecystographic agents. Despite the fact that albumin itself carries a net negative charge at physiological pH, it is frequently proposed (e.g. 206, 124) that albumin binds negatively charged ligands in preference to ligands carrying a positive charge. However, albumin is also able to bind several positively charged drugs with an association constant comparable to that of most negatively charged drugs (table 3). In this connection it is interesting to note that very high binding constants have been reported for the antimalarial drug pamaquine (186). Furthermore, albumin is able to bind various inorganic ligands (table 4). It can be seen from Table 4 that binding of several of the metal ions apparently is best described by assuming binding to a relatively great number of weak binding sites.

In most cases binding data have been analyzed according to the Scatchard model (233) assuming that the ligand in question is bound to classes of identical, independent binding sites. Another way of analyzing binding data is by using a stepwise equilibrium model. The binding of medium-chain (7) and long-chain (8) fatty acids especially has been analyzed in this way. For example, the first five association constants (K) calculated for the binding of palmitate are as follows: 6.2×10^7 M^{-1}, K_2 =

TABLE 1

*Binding of endogenous substances to albumin**

Ligand	n_1	K_{ass_1}, M^{-1}	n_2	K_{ass_2}, M^{-1}	Albumin	pH	Temperature, °C	Reference
Bilirubin	1	1.4×10^8	2	5×10^5	HSA[f]	7.4	37	108
Bilirubin	1	5.5×10^7	1	4.4×10^6	HSA[d]	7.4	37	29
Haemin	1	5.0×10^7			HSA[d]	7.5	23	10
Haemin	1	1.1×10^8			HSA[f]	7.0	24	1
Palmitate	2	6.0×10^7	5	3.0×10^6	HSA[d]	7.45	23	82
Linoleate	2	1.3×10^7	5	2.5×10^6	HSA[d]	7.45	23	82
Oleate	2	1.1×10^8	5	4.0×10^6	HSA[d]	7.45	23	82
L-Tryptophan	1	1.6×10^4			HSA[f]	7.4	2	164
L-Thyroxine	1	1.6×10^6	3	6×10^4	HSA[d]	7.4	24	256
Progesterone	1	3.6×10^5	8	6×10^3	HSA[d]	7.4	4	289
Testosterone	1	2.38×10^4			HSA[f]	7.4	25	279
Estradiol	1	1.0×10^5			HSA[f]	7.4	5	57
Aldosterone	1	3.2×10^3			HSA[f]	7.4	37	221
Cortisol	2	5.0×10^3			HSA[f]	7.4	37	296
Cholate	1	3.2×10^4	7	2×10^3	HSA[f]	7.4	37	40
Taurocholate	1	1.2×10^4			BSA[d]	7.4	25	84
Lysolecithin	1	4.3×10^4			BSA[f]	Isoionic pH	20	121,122

* n_1 and n_2 are the number of binding sites in the first and second binding class, respectively, and K_{ass_1} and K_{ass_2} are the corresponding Scatchard association constants; HSA, human serum albumin; BSA, bovine serum albumin; f, nondefatted albumin; d, defatted albumin.

TABLE 2

*Binding of negatively charged and electrostatic neutral drugs to nondefatted human serum albumin at pH 7.4**

Drug	n_1	K_{ass_1}, M^{-1}	n_2	K_{ass_2}, M^{-1}	Method	Temperature, °C	Reference
Warfarin	1	2.5×10^5	2	1.1×10^4	Fluorescence	22	261
Warfarin	2	8.9×10^4	4	6.7×10^3	Equilibrium dialysis	27	77
Warfarin	2	1.5×10^5	5	1.5×10^3	Dynamic dialysis	37	37
Warfarin	1	2.3×10^5	4	5.9×10^3	Equilibrium dialysis + ultrafiltration	37	272
Dicoumarol	1	2.9×10^6	1	1.8×10^5	Circular dichroism	ca. 20†	204
Dicoumarol	2	2.2×10^6	7	1.3×10^4	Equilibrium dialysis	27	77
Dicoumarol	3	7.7×10^5			Equilibrium dialysis	ca. 20†	49
Dicoumarol	3	3.5×10^5			3 different methods	20	51
Salicylate	1	7.1×10^4	4	3.3×10^3	Gel frontal analysis chromatography	25	117
Salicylate	1	2.2×10^5	5	1.6×10^3	Dynamic dialysis	37	37
Salicylate	2	1.3×10^5	2	2.9×10^3	Equilibrium dialysis	20	99
Phenylbutazone	1	2.37×10^5	2	4.56×10^4	Circular dichroism	ca. 20†	223
Phenylbutazone	1	1×10^5	2	4×10^4	Equilibrium dialysis	ca. 20†	48
Phenylbutazone	3	2.5×10^5	4	1.3×10^3	Dynamic dialysis	37	37
Phenylbutazone	1	2.3×10^5	4	5.6×10^3	Equilibrium dialysis + ultrafiltration	37	272
Digitoxin	1	4.3×10^4			Equilibrium dialysis	37	27
Digitoxin	1	6.9×10^4			Equilibrium dialysis	20	151
Iopanoate	1	6.7×10^6	1	1.5×10^6	Ultrafiltration	20–25	180
Iopanoate	3	7×10^5	40	9×10^2	Equilibrium dialysis	22–25	140
Iophenoxate	1	7.7×10^7	1	3.8×10^5	Ultrafiltration	20–25	180
Clofibrate	2	2.5×10^4	8	4.7×10^2	Equilibrium dialysis	ca. 20†	189
Chlorophenoxyisobutyrate	1	1.3×10^5	16	1.5×10^3	Equilibrium dialysis	37	254‡
Chlorophenoxyisobutyrate	1	3.3×10^5	4	3.9×10^3	Equilibrium dialysis + ultrafiltration	37	272
Halofenate	3	1.6×10^5	3	7.0×10^3	Equilibrium dialysis	37	254‡
Tolbutamide	2	2.2×10^5	8	1.7×10^2	Dynamic dialysis	37	37, 55
Indomethacin	1	1.0×10^6	4	1.0×10^5	Equilibrium dialysis	20	99
Indomethacin	1	3.0×10^5	7	1.4×10^4	Ultrafiltration	37	157
Furosemide	1	1.7×10^5	4–5	9.6×10^3	High-performance liquid chromatography	37	236
Sulfaphenazole	1	9.2×10^4	5	1.1×10^3	Dynamic dialysis	37	37
Sulfaethidole	1	1.5×10^5	3	1.6×10^3	Equilibrium dialysis	25	109
Fusidic acid	3	7.8×10^4			Antibiotic activity	37	88
Chlorothiazide	2	3.07×10^4			Equilibrium dialysis	37	26
Chlorpropamide	2	4.5×10^4	8	1.7×10^2	Dynamic dialysis	37	55
Glibenclamide	2	7.7×10^5			Equilibrium dialysis	37	55
Diazepam	1	4.9×10^5			Gel filtration	22	182
Chlorazepate	1	1.3×10^4			Microcalorimetry	30	54
Tolazamide	1	8.7×10^4	3	1.5×10^3	Dynamic dialysis	37	55
Cinchophen	2	1.4×10^5			Ultrafiltration	ca. 20†	181
Novobiocin	1	5.5×10^5	1	3.7×10^4	Equilibrium dialysis + circular dichroism	30	21
Dansylglycin	1	4.6×10^5			Fluorescence	ca. 20†	48
Camptothecin	1	8.0×10^6			Fluorescence	ca. 20†	50
Acenocoumarin	1	1.96×10^5	4	6.0×10^3	Equilibrium dialysis + ultrafiltration	37	272
Ibuprofen	1	2.73×10^6	6–7	1.95×10^4	Equilibrium dialysis	37	290

* n_1 and n_2 are the number of binding sites in the first and second binding class, respectively, and K_{ass_1} and K_{ass_2} are the corresponding Scatchard association constants.

† Room temperature (?).

‡ Results obtained with defatted albumin.

$2.3 \times 10^7\ M^{-1}$, $K_3 = 1.2 \times 10^7\ M^{-1}$, $K_4 = 3.1 \times 10^6\ M^{-1}$, $K_5 = 1.5 \times 10^6\ M^{-1}$. For a detailed discussion of the relation between site binding constants ("Scatchard model") and stoichiometric binding constants (stepwise equilibrium model), see Klotz and Hunston (123).

When comparing binding data from different laboratories several precautions must be taken. It is important to note the fatty acid content of the albumin preparations, because fatty acids bound to albumin are able to influence the simultaneous binding of other ligands. The fatty acid content varies from very low concentrations (of the order of 0.1 mole per mole of protein) to as much

TABLE 3

*Binding of positively charged drugs to albumin**

Drug	n_1	K_{ass_1}, M^{-1}	Albumin	pH	Method	Temperature, °C	Reference
Chlorpromazine	4	4.2×10^4	HSA[d]	7.4	Difference spectrophotometry	25	74, 97
Chlorpromazine	2	1.9×10^5	HSA[f]	7.4	Fluorescence	20	240
Triflupromazine	4	5.5×10^4	HSA[d]	7.4	Difference spectrophotometry	25	97
Promazine	1–2	8.5×10^4	HSA[f]	7.4	Fluorescence	20	240
Imipramine	1–2	2.39×10^4	HSA[f]	7.4	Fluorescence	20	240
Desipramine	1–2	7.02×10^4	HSA[f]	7.4	Fluorescence	20	240
Quinine	1	7.5×10^3	HSA[f]	7.4	Ultrafiltration	20	199
Quinidine	2	1.4×10^3	HSA[f]	7.4	Equilibrium dialysis	20	193
Procaine	2	3.1×10^3	HSA[f]	7.0	Equilibrium dialysis	4	232
Lidocaine	2	1.3×10^5	HSA[f]	7.0	Equilibrium dialysis	4	232
Mepivacaine	2	2.5×10^5	HSA[f]	7.0	Equilibrium dialysis	4	232
Pamaquine	1	6.4×10^7	BSA[f]	7.4	Fluorescence	25	186

* n_1 is the number of binding sites in the first binding class and K_{ass_1} is the corresponding Scatchard association contant; no secondary binding classes were reported on in the studies cited except in the case of pamaquine [$n_2 = 1$; $K_{ass_2} = 3.1 \times 10^6$ M^{-1}, and $n_3 = 1$; $K_{ass_3} = 1.9 \times 10^5$ M^{-1} (186)]. HSA, human serum albumin; BSA, bovine serum albumin; f, nondefatted albumin; d, defatted albumin.

TABLE 4

*Binding of inorganic ions to nondefatted albumin**

Ligand	n_1	K_{ass_1}, M^{-1}	n_2	K_{ass_2} M^{-1}	Albumin	pH	Temperature, °C	Reference
Cl^-	1	7.2×10^2	4	6.1×10^1	HSA	Isoionic pH	25	234
I^-	1	6.15×10^3	4	6.7×10^2	HSA	Isoionic pH	25	234
SCN^-	1	3.35×10^4	4	7.8×10^2	HSA	Isoionic pH	25	234
Zn^{++}	17†	5.7×10^2			HSA	6.07	25	280
Cd^{++}	17	1.3×10^3			BSA	5.95	25	280
Mn^{++}	1	2.4×10^4	5	5.0×10^2	HSA	7.0	4?	188
Cu^{++}	1	9×10^6			BSA	7.0	25	206
Ni^{++}	1	3×10^5			HSA	7.4	4	42
Co^{++}	2	6.5×10^3	23	1.6×10^2	HSA	7.1	25	187
Ca^{++}	12	10^2			HSA	7.4	37	200
Mg^{++}	12	10^2			HSA	7.4	37	200, 201

* n_1 and n_2 are the number of binding sites in the first and second binding class, respectively, and K_{ass_1} and K_{ass_2} are the corresponding Scatchard association constants; HSA, human serum albumin; BSA, bovine serum albumin.

†Österberg (198) reported $n_1 = 1$, $n_2 = 1$, and high K_{ass} values (BSA).

as 2 moles to 1 mole of albumin. Binding of ligands to albumin has been studied with different techniques. However, since different methods do not have the same sources of error, a binding isotherm obtained by one technique is not necessarily comparable to that acquired by another method, which results in differences in the Scatchard constants calculated (some examples are given in tables 2 and 3). Furthermore, experiments have shown that binding data can be influenced by factors such as albumin concentration, pH, temperature, and type and concentration of salts in the solution studied. Even different batches of albumin, although isolated and purified according to the same principles, can result in different binding parameters. Therefore, rather wide limits have to be accepted, when binding results obtained in different laboratories are compared, before the conclusion of significant differences is drawn.

III. Structure of Albumin

A. *Human Serum Albumin*

All serum albumins examined consist of a single polypeptide chain. Studies on the complete amino acid sequence of human serum albumin were published simultaneously in 1975 by Brown and coworkers (11) and Meloun et al. (168 and fig. 1). Later on, the proposal of the amino acid sequence from Brown's laboratory was modified (35) (fig. 2). Meloun et al. (168) and Brown (35) found that human albumin contains 585 amino acid residues. Both models place the lone sulfhydryl group and the lone tryptophan residue in positions 34 and 214, respectively. Although the sequences proposed display a remarkable resemblance, there are differences. In the following positions the sequences diverge (cf. figs. 1 and 2): 37–38, 170, 279–282, 295, 344–345, 352, 364–365, 367–368, 370, 393, 455, 464–465, and 501. In a more recent publication (36) Brown proposed changes in the sequence of the segment 279–282 so that it is identical with that of Meloun et al. (168). In positions 266–269, 382, and 397 Brown (35), in contrast to Meloun et al. (168), has not decided whether the residues are found in their acidic or amide form.

Several papers have appeared determining the amino acid sequence of segments of human albumin. Behrens et al. (11) and Meloun et al. (168) commented and compared the results of their studies with those published

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                5                  10                  15
  1  Asp-Ala-His-Lys-Ser-Glu-Val-Ala-His-Arg-Phe-Lys-Asp-Leu-Gly-
 16  Glu-Glu-Asn-Phe-Lys-Ala-Leu-Val-Leu-Ile-Ala-Phe-Ala-Gln-Tyr-
 31  Leu-Gln-Gln-Cys-Pro-Phe-Glu-Asp-His-Val-Lys-Leu-Val-Asn-Glu-
 46  Val-Thr-Glu-Phe-Ala-Lys-Thr-Cys-Val-Ala-Asp-Glu-Ser-Ala-Glu-
 61  Asn-Cys-Asp-Lys-Ser-Leu-His-Thr-Leu-Phe-Gly-Asp-Lys-Leu-Cys-
 76  Thr-Val-Ala-Thr-Leu-Arg-Glu-Thr-Tyr-Gly-Glu-Met-Ala-Asp-Cys-
 91  Cys-Ala-Lys-Glu-Gln-Pro-Glu-Arg-Asn-Glu-Cys-Phe-Leu-Gln-His-
106  Lys-Asp-Asp-Asn-Pro-Asn-Leu-Pro-Arg-Leu-Val-Arg-Pro-Glu-Val-
121  Asp-Val-Met-Cys-Thr-Ala-Phe-His-Asp-Asn-Glu-Glu-Thr-Phe-Leu-
136  Lys-Lys-Tyr-Leu-Tyr-Glu-Ile-Ala-Arg-Arg-His-Pro-Tyr-Phe-Tyr-
151  Ala-Pro-Glu-Leu-Leu-Phe-Phe-Ala-Lys-Arg-Tyr-Lys-Ala-Ala-Phe-
166  Thr-Glu-Cys-Cys-Glu-Ala-Ala-Asp-Lys-Ala-Ala-Cys-Leu-Leu-Pro-
181  Lys-Leu-Asp-Glu-Leu-Arg-Asp-Glu-Gly-Lys-Ala-Ser-Ser-Ala-Lys-
196  Gln-Arg-Leu-Lys-Cys-Ala-Ser-Leu-Gln-Lys-Phe-Gly-Glu-Arg-Ala-
211  Phe-Lys-Ala-Trp-Ala-Val-Ala-Arg-Leu-Ser-Gln-Arg-Phe-Pro-Lys-
226  Ala-Glu-Phe-Ala-Glu-Val-Ser-Lys-Leu-Val-Thr-Asp-Leu-Thr-Lys-
241  Val-His-Thr-Glu-Cys-Cys-His-Gly-Asp-Leu-Leu-Glu-Cys-Ala-Asp-
256  Asp-Arg-Ala-Asp-Leu-Ala-Lys-Tyr-Ile-Cys-Glu-Asn-Gln-Asp-Ser-
271  Ile-Ser-Ser-Lys-Leu-Lys-Glu-Cys-Cys-Glu-Lys-Pro-Leu-Leu-Glu-
286  Lys-Ser-His-Cys-Ile-Ala-Glu-Val-Glu-Asn-Asp-Glu-Met-Pro-Ala-
301  Asp-Leu-Pro-Ser-Leu-Ala-Ala-Asp-Phe-Val-Glu-Ser-Lys-Asp-Val-
316  Cys-Lys-Asn-Tyr-Ala-Glu-Ala-Lys-Asp-Val-Phe-Leu-Gly-Met-Phe-
331  Leu-Tyr-Glu-Tyr-Ala-Arg-Arg-His-Pro-Asp-Tyr-Ser-Val-Val-Leu-
346  Leu-Leu-Arg-Leu-Ala-Lys-Thr-Tyr-Glu-Thr-Thr-Leu-Glu-Lys-Cys-
361  Cys-Ala-Ala-His-Asp-Pro-Tyr-Glu-Cys-Ala-Ala-Lys-Val-Phe-Asp-
376  Glu-Phe-Lys-Pro-Leu-Val-Glu-Glu-Pro-Gln-Asn-Leu-Ile-Lys-Gln-
391  Asn-Cys-Glu-Leu-Phe-Glu-Gln-Leu-Gly-Glu-Tyr-Lys-Phe-Gln-Asn-
406  Ala-Leu-Leu-Val-Arg-Tyr-Thr-Lys-Lys-Val-Pro-Gln-Val-Ser-Thr-
421  Pro-Thr-Leu-Val-Glu-Val-Ser-Arg-Asn-Leu-Gly-Lys-Val-Gly-Ser-
436  Lys-Cys-Cys-Lys-His-Pro-Glu-Ala-Lys-Arg-Met-Pro-Cys-Ala-Glu-
451  Asp-Tyr-Leu-Ser-Val-Val-Leu-Asn-Gln-Leu-Cys-Val-Leu-Glu-His-
466  Lys-Thr-Pro-Val-Ser-Asp-Arg-Val-Thr-Lys-Cys-Cys-Thr-Glu-Ser-
481  Leu-Val-Asn-Arg-Arg-Pro-Cys-Phe-Ser-Ala-Leu-Glu-Val-Asp-Glu-
496  Thr-Tyr-Val-Pro-Lys-Gln-Phe-Asn-Ala-Glu-Thr-Phe-Thr-Phe-His-
511  Ala-Asp-Ile-Cys-Thr-Leu-Ser-Glu-Lys-Glu-Arg-Gln-Ile-Lys-Lys-
526  Gln-Thr-Ala-Leu-Val-Glu-Leu-Val-Lys-His-Lys-Pro-Lys-Ala-Thr-
541  Lys-Glu-Gln-Leu-Lys-Ala-Val-Met-Asp-Asp-Phe-Ala-Ala-Phe-Val-
556  Glu-Lys-Cys-Cys-Lys-Ala-Asp-Asp-Lys-Glu-Thr-Cys-Phe-Ala-Glu-
571  Glu-Gly-Lys-Lys-Leu-Val-Ala-Ala-Ser-Gln-Ala-Ala-Leu-Gly-Leu
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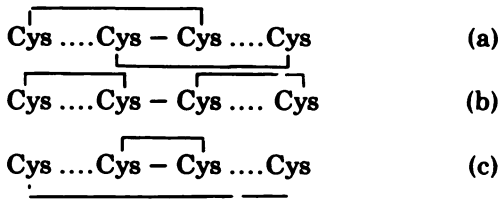
FIG. 1. Complete amino acid sequence of human serum albumin as represented by Meloun et al. (168). [Reproduced by permission of the authors and F.E.B.S. (Fed. Eur. Biochem. Soc.) Lett.]

before 1975. However, other papers appeared in 1975 and later. Gambhir et al. (76) investigated the sequence of a fragment of human albumin and suggested that it is composed of 110 amino acid residues. The segment includes the lone tryptophan residue, which bears the number 86. By identifying this position with number 214 of Meloun et al. (168) and Brown (35), the best accordance is obtained when the fragment is compared with section 124–238 of the proposals of the American and Czech groups of investigators. The only divergent assignment of Meloun et al. (168) and Brown (35) included in the fragment is position 170. Gambhir et al. (76) suggest, in accordance with Brown (35) but in disagreement with Meloun et al. (168), that glutamine and not glutamic acid is placed in that position.

Walker (281, 282) also reported on sequences of albumin fragments. He determined (281) the sequence of a small section of the protein acetylated by acetylsalicylic acid to be: Leu-Lys-Cys-Ala-Ser-Leu-Gln-Lys. This peptide corresponds completely to the residues 198–205 of Meloun et al. (168) and of Brown (35). Walker (282) also studied a fragment containing 90 amino acids of the C-terminal end of human albumin. The sequence is in remarkable agreement with the corresponding proposals of the complete models (figs. 1 and 2). However, there are two small differences. In agreement with Brown (35), Walker (282) found glutamic acid instead of glutamine at position 501. Both Meloun et al. (168) and Brown (35) suggest aspartic acid in position 550 in contrast to Walker (282), who proposes asparagine.

The three-dimensional structure of human albumin is influenced and stabilized to a great extent by the 17 disulfide bonds. A characteristic feature is the presence of two neighboring cysteinyl residues throughout the sequence. Saber et al. (229) examined the distribution of the disulfide bonds in human albumin and found that they were arranged as groups of two pairs of adjacent disulfide bonds uniformly distributed in the protein molecule except in the part closest to the N-terminal end, which contained only one disulfide bridge. Brown (35) modified the disulfide pattern originally suggested by his group (11) to be in full accordance with that of Saber et al. (229). The characteristic pattern of the disulfide bonds of human albumin is analogous to that of bovine albumin (fig. 3). Saber et al. (229) mentioned that a group of two disulfide bonds can be arranged in the following three ways:



In the study cited, possibility c could be excluded, whereas a distinction between a and b demanded further investigation. Brown (35) arranged the groups of disulfide bonds according to possibility a.

B. Variants of Human Serum Albumin

The existence of a great number of albumin "variants" has been suggested in the literature on the basis of distinct electrophoretic mobilities (71). However, changes in electrophoretic mobilities could also be caused by other means such as protein aggregation, ligand binding, deamidation, and oxidation. On the other hand, there is the possibility that two albumin variants could comigrate. The best way to identify albumin variants probably is to determine differences in the amino acid sequence of the proteins. A few studies identify variants in this way. Winter et al. (293) found two albumins, albumin Oliphant in families of German descent and albumin Ann Arbor in families of Danish descent, which probably are identical but different from the most common form of albumin, called albumin A (figs. 1 and 2). In the variants, glutamic acid (293) in position 570 (71) of albumin A has been replaced by lysine (293). Franklin et al. (71) found an albumin variant, which they named Mexico-2, among Indians in Mexico and in the Southwestern United States. This albumin variant differs from albumin A in position 550, where the aspartic acid is replaced by glycine in albumin Mexico-2. Another variant, Mexico-1, was found in the same area. However, at present the relation between the Mexico-1 albumin and the other two albumins remains to be clarified.

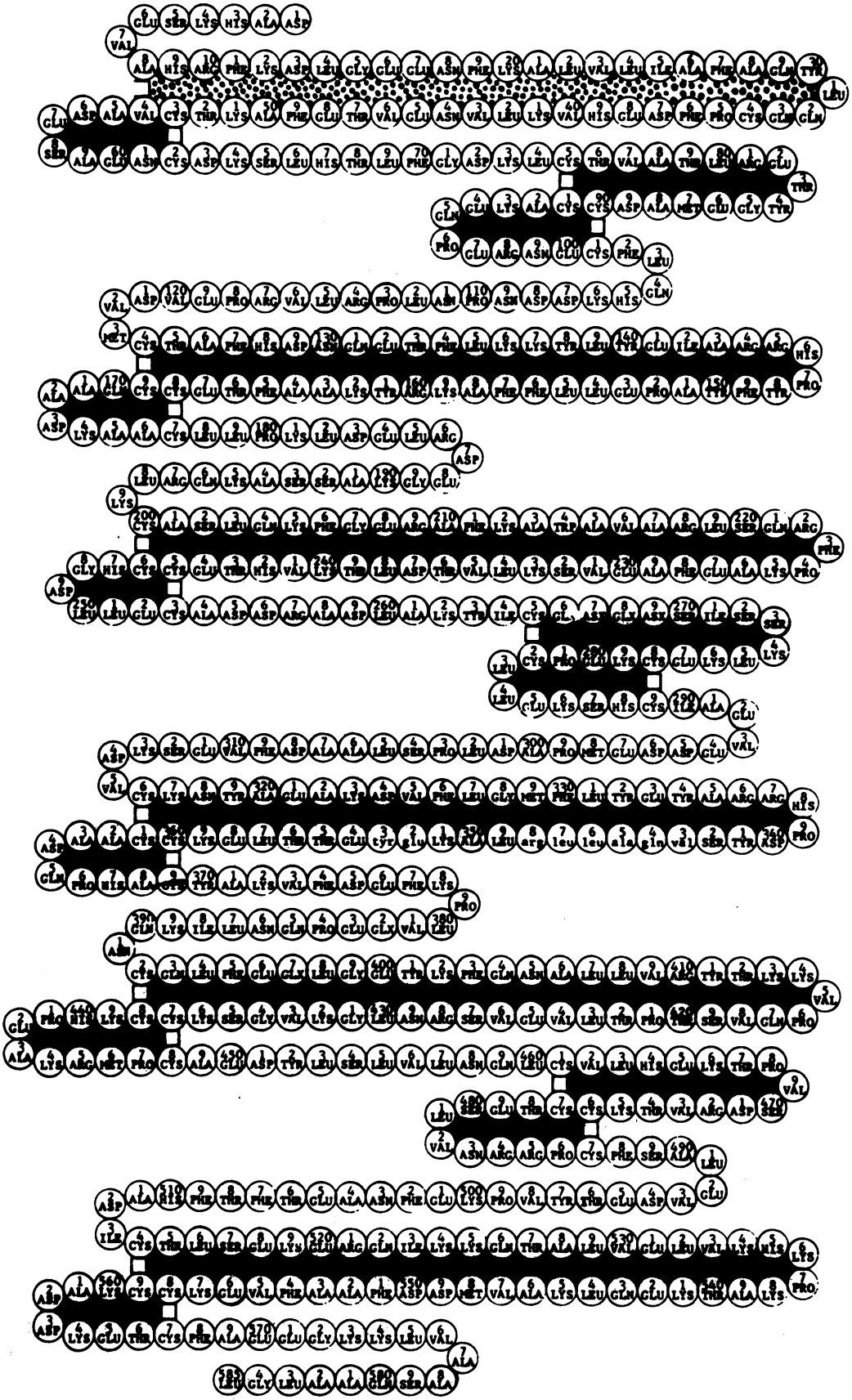


FIG. 2. Amino acid sequence of human serum albumin, displayed in a model showing the proposed linking of cysteines to form multiple double loops as proposed by Brown (35). Residues assigned by analogy with bovine serum albumin are shown in lower case letters (positions 343–348 and 352–353). (Reproduced by permission of the author and Pergamon Press.)

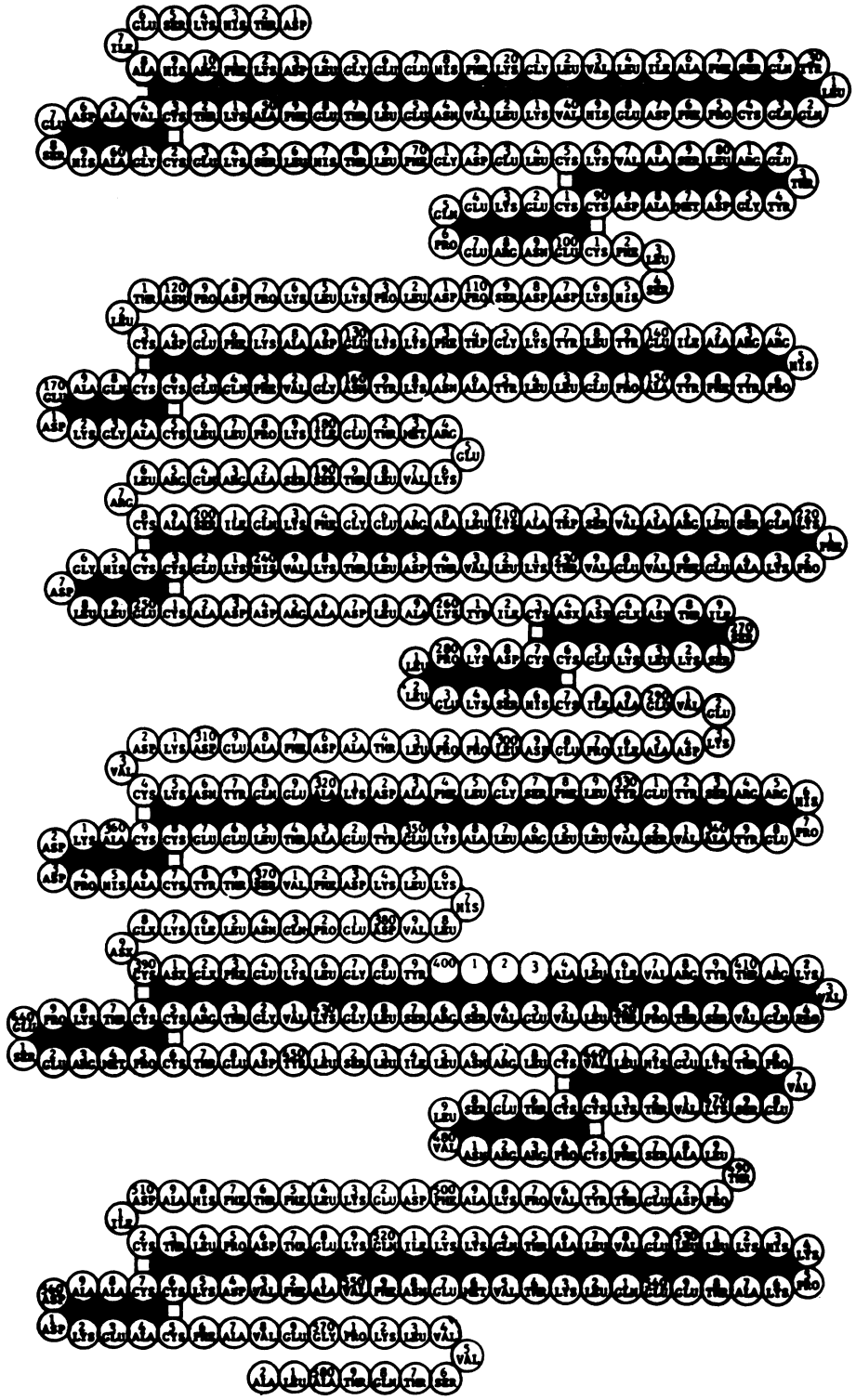


FIG. 3. Amino acid sequence of bovine serum albumin, displayed in a model showing the proposed linking of cysteines to form multiple double loops as proposed by Brown (36). The sequence is complete except for residues 400–403. According to Reed et al. (217) these residues are Gly-Phe-Gln-Asn. (Reproduced by permission of the author and Pergamon Press.)

C. *Bovine Serum Albumin*

Brown has most intensively studied the structure of bovine serum albumin and the primary structure has been presented and discussed several times (33–36). The latest version of the sequence was published in 1977 (36) and is depicted in figure 3. The sequence of all of the 582 amino acid residues, except 4 (residues 400–403), of this protein was determined by Brown. Recently, Reed et al. (217) published that the sequence of residues 400–403 is

Gly-Phe-Gln-Asn. Furthermore, these authors proposed amides in the following positions, which were left undecided by Brown (36): 388 (glutamine), 389 (asparagine), and 392 (glutamine). Amino acid residue number 391 was determined to be aspartic acid. The lone sulfhydryl group is placed in exactly the same position (no. 34) as in human albumin (figs. 1 and 2). In contrast to the human species, bovine albumin contains two tryptophan residues, placed in positions 134 and 212. Bovine albumin also contains one single and eight pairs of disulfide bonds arranged in a way analogous to that of human albumin. Of the three different ways of pairing four cysteine residues mentioned above, Brown (36), as in the case of human albumin, chose possibility a (section III A). In both species the long loop shown closest to the N-terminal end is missing a cysteine residue to close the loop. This bond probably was lost during evolution (36, 34).

Brown (35) compared the sequences of human and bovine albumin and found striking homology. Furthermore, the differences observed are mainly of a structurally conservative nature, e.g. hydrophobic amino acids are replaced by other hydrophobic amino acids and not by polar ones.

D. Common Structural Features of Human and Bovine Serum Albumin

On the basis of the distribution of the disulfide bridges and of the amino acid sequence it seems possible to regard bovine albumin as composed of three homologous domains linked together by peptide chains as represented in figure 4. In the case of this albumin species the domains are suggested (34, 35) to contain the amino acid residues 1–190 (no. 1), 191–382 (no. 2) and 383–582 (no. 3)—including interdomainal connecting segments. The domains can all be subdivided into two subdomains (A-B and C), mainly containing long loop, and an intradomainal hinge region. A subdomain can be further subdivided into three "helices" (x, y, and z) (36) as shown in figure 4. It is reasonable to assume that human albumin is composed in a similar manner.

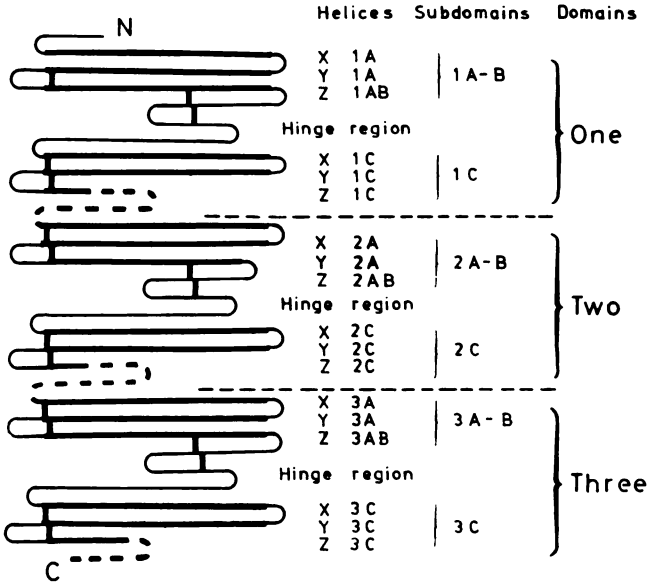


FIG. 4. Structural organization of serum albumin as proposed by Brown (35, 36). The present illustration is a modification of the typographical version represented by Brodersen (30).

Brown (34), when comparing the three domains of bovine albumin, observed that in several homologous positions the amino acids are identical. A higher degree of identity was observed between domains 1 and 2 than between domains 1-3 and 2-3. McLachlan and Walker (160) compared the sequences of the three domains of bovine and human albumin and observed a statistically significant repeat not only between the domains but also between the subdomains. When comparing the various domains, especially the sequences of the long loops, Ax-Ay and Cx-Cy (fig. 4), are comparable. Furthermore, the homology of the loops Ax-Ay and Cx-Cy inside the domains is highly significant. The homology between the domains and subdomains and the pattern of the disulfide bridges led to the view that bovine and human albumin evolved from a simple precursor consisting of 77–97 amino acids arranged as a peptide loop including a disulfide bond (34, 160).

Optical rotatory dispersion (245) and circular dichroism (216) measurements suggest that the α-helix content of bovine albumin is 54% and 68%, respectively. Brown (35) suggested that the helical parts of bovine albumin are uniformly placed in the subdomains (except at the ends of the loops where a proline residue typically is found) and in the connections between the domains (see fig. 4). Peters and Reed (210) have tried to estimate the distribution of α-helix, β-sheet, β-turns, and irregular structures in bovine albumin based on the rules of Chou and Fasman (53). The analysis indicated that, although most of the α-helices should be found in the long loops, these are not expected to be uniformly helical as suggested by Brown. For example, the calculated helix content of 1Cy and 2Cx is small and 3Ay and 3Az, surprisingly, should be completely nonhelical. McLachlan and Walker (160, 161) calculated probability profiles for the same structural elements as carried out by Peters and Reed (210) but averaged the predictions over the three domains of bovine and human albumin. First (160) they estimated the probability by the equations of Chou and Fasman (52) and Lim (146). However, the results obtained by the two methods were rather conflicting. Therefore, an alternative method was developed based on the most probable relationship between an amino acid and the secondary structure as revealed by the study of a great number of proteins with known amino acid sequence and structure (159). With this method, McLachlan and Walker found (161) that in a representative domain most of the peptide chain, including the hinge region, is structured as an α-helix and probably contains no β-sheet at all. Typical exceptions from this generalization are the outermost parts of the N- and C-terminal

ends of a domain, around the S-S bonds, the tips of the long loops in each subdomain, and also in the middle portion of the hinge regions, which probably are bend structures. Furthermore, the middle portion of the hinge region is relatively polar. McLachlan and Walker (161) found that according to their calculations the α-helix content of bovine and human albumin should be as high as 75% or more.

In summary, the above cited reports indicate that most of the residues in the long loops (except in the ends) and the sections connecting the domains probably are forming α-helices, whereas the intradomainal hinge regions are mainly nonhelical.

Brown (35, 36) suggested a model of the spatial configuration of albumin in which the subdomains are the principal elements. The three long "helices" in a subdomain run parallel to one another and form a trough by a lowering of the middle "helix" (y) (see fig. 5). The "helices" of the subdomains 1A-B, 2A-B, and 3A-B can easily be arranged in the suggested manner, while constructing a trough of the "helices" of the subdomains 1C, 2C, and 3C must require that most of the peptides connecting the domains and of the C-terminal part of a domain run parallel to the x and y "helices" (36). The "helices" are kept together by disulfide bridges (fig. 5) and by the short peptides connecting the x and y "helices." By assuming that all the amino acids in the "helices" could be arranged in such a way and that the "helices" are true α-helices, Brown found (35) that almost all the hydrophobic residues were placed between the "helices" and inside the trough, whereas the great majority of polar residues were on the outer wall of the structure. A domain is formed by adherence of two subdomains with their grooves towards each other, and three of such domains make up an albumin molecule.

The suggestion that albumin is composed of three domains is supported by low-angle X-ray scattering studies (18).

In 1960 Foster (70) suggested that albumin was composed of four domains. Brown (35) mentioned the possibility that the subdomains also can be arranged in favour of such a model. In that case subdomain 1A-B is domain no. 1, 1C and 2A-B are added to form domain no. 2, 2C and 3A-B form domain no. 3, and 3C is domain no. 4. In this model domains 2 and 3, in contrast to 1 and 4, could be the ligand binding parts of albumin (35).

The flexibility of albumin (cf. the last section of this review) is probably brought about by conformational changes in the sections connecting domains and in the relatively long and polar intradomainal hinge regions. Perhaps also the distance between the "helices" forming a domain can vary, e.g. when albumin interacts with a ligand.

In the way the cylindrical domains are formed by Brown, they have a hydrophobic interior and a polar exterior. It is not only with respect to the interior and exterior parts that the amino acid residues are nonequally distributed. Around the opening at the top of the domains especially basic and nonpolar residues are placed. Brown (35, 36) proposes that the binding site for ligands like fatty acids can be associated to these openings. The aliphatic part of a fatty acid could be inserted into the hydrophobic interior of the domain and the carboxyl group can interact electrostatically with the basic residues. Perhaps the unique ability of serum albumin to bind with a high affinity a great number of different

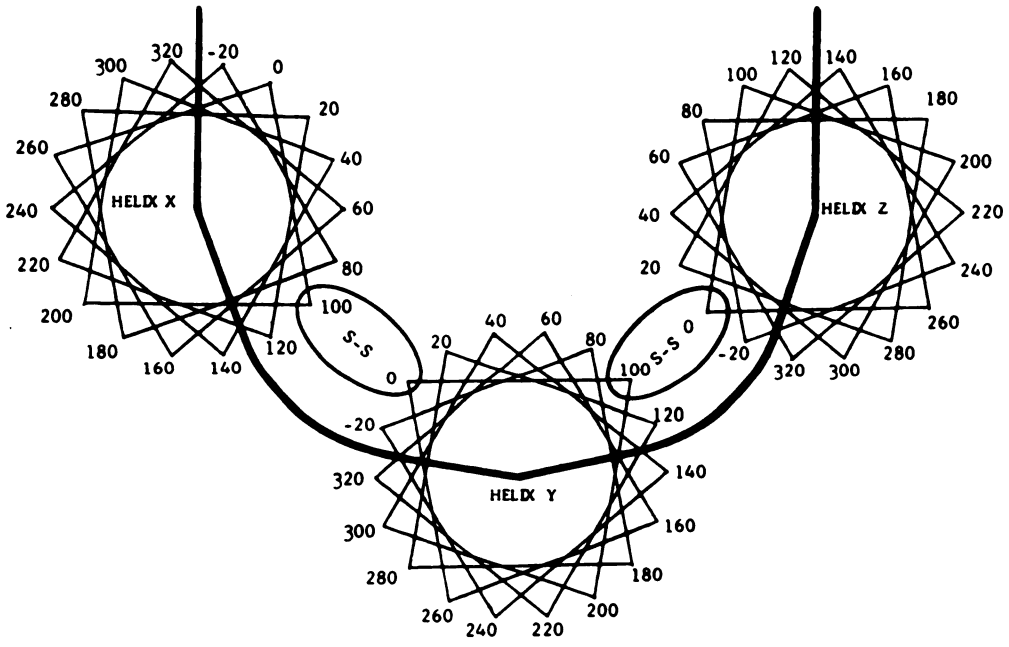


FIG. 5. Subdomain structure of serum albumin as proposed by Brown (35). (Reproduced by permission of the author and Pergamon Press.)

ligands is based on the existence of such binding sites, whereas binding of ligands with a low affinity, and probably in an unspecific manner, to areas on the surface of the protein is a common property of many proteins.

E. Species Differences in Ligand Binding Properties

The spatial configurations of serum albumins from man, cow, and other mammals probably are comparable in many respects. In accordance with this view a number of physicochemical data of human and bovine albumin are comparable [see compilation by Peters (206)]. However, since the amino acid sequences of human and bovine albumin, and probably also other albumins, are not identical, differences in certain aspects are to be expected. Although the majority of ligands seem to bind to various albumins equally well, some examples to the contrary can be given.

Brodersen (fig. 44, ref. 30) has shown that addition of fatty acids to human and bovine albumin results in an increased binding of bilirubin. The cooperativity is much more pronounced in the case of the bovine protein. Lauric acid, myristic acid, and perhaps also palmitic acid in fatty acid-albumin ratios of 1 to about 3 increase bilirubin binding to human albumin moderately. In the case of bovine albumin, addition of decanoic acid and oleic acid also results in cooperative bilirubin binding. The fatty acids apparently increase bilirubin binding by bovine albumin even when 5 to 6 moles of fatty acid per mole of protein has been added. At fatty acid-albumin ratios higher than 3 and 5 to 6 in the case of human and bovine albumin, respectively, bilirubin binding is decreased.

Woolley and Hunter (294) observed that binding of bilirubin to human albumin results in circular dichroism spectra different from those introduced by binding to bovine albumin. Addition of bilirubin to human albumin in increasing molar ratios (0.25 to 4) results in a steady increase in the intensity of a negative Cotton effect with a minimum at about 410 nm and a positive Cotton effect with a maximum at about 460 nm. In the case of bovine albumin a positive Cotton effect with maximum at 416 nm and a negative Cotton effect with minimum at 472 nm is seen at molar ratios of 0.25 to 1. At higher molar ratios (2 to 4) the band at 416 nm becomes negative. On the other hand, the band at 472 nm decreases in magnitude and becomes positive at a bilirubin-albumin molar ratio of 4. These spectral findings indicate different conformations of the primary bilirubin binding sites of the two proteins (294). Blauer et al. (16) also observed great differences between the circular dichroism spectra of bilirubin-human albumin and bilirubin-bovine albumin complexes.

Callan and Sunderman (42) calculated the first association constant for binding of Ni^{++} to serum albumin from human, rat, and rabbit to be 2 to 3 $\times$ 10^5 M^{-1} or higher. The constants calculated for binding to canine and porcine albumins were only 2.5 $\times$ 10^4 M^{-1} and 8 $\times$ 10^4 M^{-1}, respectively. Appleton and Sarkar (5) demonstrated that dog serum albumin lacks the first binding site for Cu^{++}, which is present in human, rat, and bovine albumins. The reduced ability of dog albumin to bind Ni^{++} and Cu^{++}, as compared with the other albumin species mentioned, seems to be caused by a replacement of the histidine residue by a tyrosine residue in position 3 from the N-terminal end of the protein (42, 60).

Bunn and Jandl (39) found that albumins from man and five different monkey species bound haemin tightly. This was in contrast to albumins from the cow, dog, rat, guinea pig, and rabbit.

Leatherbarrow and Dean (144) observed that human albumin, and not bovine, rabbit, horse, or sheep albumins, is bound tightly to a complex of Cibacron blue F3G A and Sepharose 4B. The authors proposed, on the basis of displacement studies, that the dye binds to bilirubin binding sites on human albumin. Iqbal and Johnson (102) also found species differences in binding of albumin to Cibacron blue-Sepharose. In their study, albumin from man but not from cow, rabbit, or chicken was bound to the dye.

Thorp (271) has mentioned other examples of species differences in ligand binding and discussed some of the implications of this variability.

Binding of ligands to various species of albumin provides an interesting possibility of elucidating the relative importance of the amino acid residues forming a binding region, as has been exemplified with the Cu^{++}- and Ni^{++}-binding site mentioned above. A different, but important, aspect is that the in vivo effects of new drugs usually are tested in small animals such as the rat before clinically testing their effects in man. Great differences in binding to the serum albumins may contribute to differences in pharmacological activity.

IV. Binding Regions of Albumin

This section presents an analysis of various high affinity binding regions of serum albumin that may be considered on the basis of mutual interaction of ligands bound simultaneously to the protein. In the following, displacement of ligand A by a ligand B according to a competitive binding scheme (i.e. $K'_A = K'_B = 0$; see Appendix) is assumed to indicate binding to the same region. On the basis of the competitive binding results reported in the literature, we propose that there are at least six binding regions on the albumin molecule. Before considering in more detail the experimental evidence for the binding regions, a sketch of the proposal is described in table 5. As discussed later in this section there is evidence that there are one or more binding regions in addition to those listed in the table. The validity of the scheme as studied by other methods and the effect of ligand-induced conformational changes will be considered in a later section.

A characteristic feature of albumin-ligand interactions seems to be the presence of one or two high affinity

TABLE 5
*Scheme for binding regions located on serum albumin**

Binding Region or Site	High Affinity Binding Site
1	Long-chain fatty acids
2	L-Thyroxine D- and L-tryptophan Chlorazepate Octanoate *p*-Iodobenzoate Chloride ion
3	Bilirubin Several dyes (e.g. phenolsulfonphthalein dyes) Iopanoate
4	Cu^{++} Ni^{++}
5	Haemin
6	Salicylate Sulfaethidole Sulfathiazole Chlorpropamide Tolbutamide Indomethacin

* In the text (section IV, F) the existence of other binding regions is discussed.

binding sites (primary sites) and a number of sites with a lower ffinity. Furthermore, at high ligand to albumin ratios several additional ligand molecules can bind with a low affinity and presumably in an unspecific manner. This type of interaction is probably a common property of many proteins.

In the case of a few ligands, e.g. dodecyl sulfate and 1-anilino-8-naphthalene sulfonate, it is not possible to single out one high affinity site, but the ligands appear to be bound to albumin to 8 (213) and 4 (127) sites, respectively, with the same association constant. It is reasonable to assume that at least some of these sites are placed in the binding regions listed in table 5 (263, 133).

A. Binding Region 1

In 1958 Goodman (82) concluded that the two sites in the first binding class of fatty acids (laurate, myristate, palmitate, stearate, oleate, and linoleate), in contrast to the sites in the second binding class, are "rather specifically constructed." The specificity of the high affinity sites of long-chain fatty acids was supported by Spector et al. (254) and Spector (251), who emphasized that no conclusive evidence of competitive binding of other ligands than fatty acids to these sites had been reported in the literature.

Other binding studies indicate that the high affinity binding sites of short- and medium-chain fatty acids probably also are placed in other regions on the albumin molecule (56, 249, 126, 130). Cunningham et al. (56) studied the binding of L-tryptophan to human serum albumin in the presence of various fatty acids. They observed that tryptophan binding was more reduced by the presence of low concentrations of laurate than by equimolar amounts of palmitate or oleate. Cunningham et al. (56) suggested that the primary binding class of medium-chain fatty acids corresponds to sites in the secondary class, but is distinct from the primary class, of long-chain fatty acids. Soltys and Hsia (249) investigated the displacing effect of various fatty acids on the binding of the spin-labelled ligand GABA-DNB-SL (shown in fig. 6), which binds to the same region as bilirubin on human serum albumin. On the basis of the different displacing effect exhibited by the fatty acids, the existence of two separate sets of high affinity fatty acid binding sites was suggested, namely, one for fatty acids of chain length less than 10 and one for fatty acids with more than 10 carbon atoms, respectively.

Koh and Means (126) investigated the inhibitory effect of fatty acids of various chain length (butyrate to decanoate) on the interaction of *p*-nitrophenylacetate and human serum albumin. They found that the fatty acids studied are bound to the same binding site, which is characterized by a capacity to accommodate nine methylene groups. Furthermore, Koh and Means (126) stated that fatty acids larger than decanoate interact with other sites.

Recently, Kragh-Hansen (130) found supporting evidence for placing the primary binding sites of long-chain fatty acids (C_{16}–C_{18}) (region 1), medium-chain fatty acids (C_8–C_{10}) (region 2) and phenol red (region 3) in three different binding regions. The binding characteristics of dodecanoic acid and myristic acid seemed to be intermediate to those of the fatty acids with longer and shorter chain lengths, respectively.

De Miranda et al. (59) studied the binding of six fatty acids with increasing carbon chain length (i.e. propionic acid to caprylic acid) to bovine serum albumin. These authors suggested the existence of a separate binding site for fatty acids containing four or less carbon atoms.

On the basis of the reports cited one may conclude that on the albumin molecule the two high affinity sites for long-chain fatty acids are placed in one region, whereas that of medium-chain fatty acids is placed in another region (probably the same as that of tryptophan, see below). Furthermore, in the case of bovine albumin, the existence of a separate site for very short-chain fatty acids (less than five carbon atoms) has been suggested.

Recently, Berde et al. (14) published a study of the binding of various preparations of long-chain fatty acids to human serum albumin. They suggested that the first two fatty acid molecules bind side-to-side in an antiparallel fashion to the same region on the albumin molecule.

B. Binding Region 2

Binding of both L-tryptophan and L-thyroxine to human serum albumin is characterized by one high affinity binding site but with association constants differing by

two orders of magnitude (table 1). Tritsch and Tritsch (274) showed that these physiological substances at pH 7.35 are bound competitively to human serum albumin.

Cunningham et al. (56) studied the binding of L-tryptophan to defatted albumin at pH 7.4 in the presence of various amounts of fatty acids. The presence of 1 mole of palmitate or oleate per mole of human serum albumin had little effect on the binding of the amino acid. However, addition of a further mole of the fatty acids reduced $n \times K$ for the binding of tryptophan to about the half. (n and K are the number of binding sites and the corresponding association constants, respectively). McMenamy (163) also studied the displacement of albumin-bound L-tryptophan by fatty acids at pH 7.4. Fatty acids at a molar ratio of 1:1 with albumin had no significant effect on the amino acid binding. At a fatty acid ratio of 2:1 a 15% reduction of n and K of tryptophan binding was observed. These observations are in agreement with those of Cunningham et al. (56). McMenamy (163) furthermore observed that addition of 4 moles of fatty acids per mole of albumin essentially blocked the tryptophan binding.

These observations (56, 163) can be explained by assuming that the 1 and 2 binding sites for long-chain fatty acids are placed in another part of human serum albumin than the part including the primary site of tryptophan. The reduced tryptophan binding in the presence of 2 moles of long-chain fatty acids could be accounted for by conformational changes of the protein (56). Another explanation of the reduced tryptophan binding is based on the heterogeneity of the fatty acid-albumin complexes (56). At a molar ratio of fatty acids to albumin of 2:1 most protein molecules associate 2 ligand molecules. However, a significant proportion of the albumin molecules will bind less or more than 2 fatty acid molecules (252). Therefore, the reduced amino acid binding could be explained by assuming competitive binding, when more than 2 fatty acid molecules are bound per albumin molecule. Cunningham et al. (56) concluded that inhibition of tryptophan binding occurs when long-chain fatty acids bind to their secondary sites. The results of McMenamy (163) show that the 3 and/or 4 binding site, in a stepwise scheme, of long-chain fatty acids is close to or identical with the primary binding site of L-tryptophan.

King and Spencer (120) have shown that L-tryptophan, D-tryptophan, and octanoate bind to only one high affinity binding site on bovine albumin. Furthermore, they found that D-tryptophan competitively displaced the two other ligands from the protein, suggesting that all three ligands bind to the same primary site of bovine albumin.

Tabachnick (263) calculated the association constants for binding of several organic anions to the high affinity binding sites of L-thyroxine of human albumin. [Tabachnick originally suggested two high affinity sites for thyroxine (263). In later studies Tabachnick (264) and Steiner et al. (256) found one primary site for thyroxine binding.] The binding constants of oleate, linoleate, and palmitate were about 1.3×10^6 M^{-1}, 5.6×10^5 M^{-1}, and 2.0×10^5 M^{-1}, respectively. The experiments with oleate and linoleate were carried out with nondefatted albumin, whereas the effect of palmitate was studied with nondefatted as well as defatted albumin. The binding constants of the fatty acids to the thyroxine sites are comparable to those in the secondary binding class of the fatty acids (table 1). Salicylate was bound to the thyroxine sites with a constant of $4–16 \times 10^3$ M^{-1} also suggesting an association between the high affinity sites of thyroxine and a secondary binding site of this ligand (table 2). Furthermore, the results indicated that a primary binding site of dodecyl sulfate and 2,4-dinitrophenol is placed in the same region as that of thyroxine (263).

More recently, Tabachnick and Korcek (266) proposed that *p*-iodobenzoate and thyroxine compete for the same high affinity binding site on human serum albumin.

Coassolo et al. (54) studied the binding of L-tryptophan and chlorazepate (a benzodiazepine) to human serum albumin by microcalorimetry and concluded that the two ligands are specific competitors for the same primary binding site.

Sjödin (241) observed changes in the ellipticity of albumin-bound diazepam in the presence of various concentrations of oleate. The ellipticity decreased linearly when the fatty acid to human serum albumin ratio was increased from 0.5 to about 2.5. At higher ratios, apparently, the ellipticity remained constant. The decrease in ellipticity was explained as liberation of diazepam, and it was suggested that the displacement of the ligand by oleate was not due to competitive binding but was caused by allosteric effects. If diazepam is bound to the same site as that of tryptophan, it is surprising that the binding of diazepam, in contrast to tryptophan, is reduced by fatty acids at the lowest ratios mentioned. However, this discrepancy probably can be explained by the different methods used. Sjödin (241) studied the binding of diazepam by circular dichroism, whereas Cunningham et al. (56) and McMenamy (163) used ultrafiltration and equilibrium dialysis, respectively. Other ultrafiltration experiments have shown that diazepam bound to human albumin is displaced by addition of 4.7 moles of laurate per mole of protein (275).

Krasner and McMenamy (135) and McMenamy (162) observed that chloride ions are inhibitors of the binding of acetyl-L-tryptophan. McMenamy (162) calculated that the association constant of binding of a chloride ion to the acetyl-L-tryptophan binding site of human albumin was 4.5×10^2 M^{-1} at 18°C. Since that figure is more in accordance with K_1 (7.2×10^2 M^{-1}) than K_2 (6.1×10^1 M^{-1}) of chloride ion binding (cf. table 4), the two ligands could share a common primary binding site. Acetyl-L-tryptophan is assumed to be bound to the same site as that of L-tryptophan.

It seems reasonable, on the basis of the above-mentioned data, to suggest that the high affinity binding site of L-thyroxine, D- and L-tryptophan, octanoate, chlor-

azepate, *p*-iodobenzoate, and chloride ions as well as one high affinity site of dodecyl sulfate and 2,4-dinitrophenol are closely associated. The literature also proposes that a secondary binding site of long-chain fatty acids and of salicylate is related to this region.

C. Binding Region 3

Unconjugated bilirubin is bound to one high affinity binding site and one or two sites with a lower affinity on albumin (table 1). Several laboratories, with different techniques, have studied the influence of long-chain fatty acids on the bilirubin binding (270, 31, 294, 196, 255). All investigators agree that in pure albumin solutions the presence of 4 moles of fatty acid per mole of albumin causes no significant liberation of bilirubin from its primary binding site. However, at higher fatty acid to human albumin ratios the binding of bilirubin is reduced.

Kragh-Hansen et al. (133), with an ultrafiltration technique (134), have shown that bilirubin and bromophenol blue competitively displace phenol red from human serum albumin. As in the case of bilirubin, addition of oleate and palmitate at a fatty acid-albumin molar ratio of approximately 4 did not significantly reduce the binding of phenol red (133). Kamisaka et al. (111) studied the binding of bilirubin and several dyes to human and bovine serum albumin by circular dichroism methods. Results obtained with a bilirubin-human serum albumin ratio of 1:1 indicated competitive binding of vasoflavine, indocyanine green, iodipamide, bromocresol green, flavaspidic acid, and phenol red, but not of sulfobromophthalein or rose bengal, to the first binding site of bilirubin. The same authors found that the relation between binding of bilirubin and the various dyes to bovine albumin showed a somewhat different pattern (111).

Mudge et al. (181) investigated the competitive binding of four different pairs of firmly bound ligands (iopanoate, iophenoxate, sulfobromophthalein, and bromophenol blue) to human serum albumin. Detailed computer analysis showed that iopanoate and bromophenol blue apparently bind to the same high affinity site on albumin. Iopenoxate and sulfobromophthalein bind with high affinities to two other, and different, sites. The observations of Kragh-Hansen et al. (133) and Mudge et al. (181) raise the possibility that the primary binding sites of iopanoate and bilirubin are identical, because the high affinity binding site of both ligands associated with that of bromophenol blue.

The observations cited above (133, 111, 181) indicate that the first binding site of bilirubin and several dyes, e.g. various phenolsulfonphthalein dyes, and probably also iopanoate are placed in the same region on the albumin molecule. The same region is able to associate with a high affinity dodecyl sulfate and 1-anilino-8-naphthalene sulfonate (133). Furthermore, a secondary binding site of long-chain fatty acids [in the range of the fifth to the seventh site (214)] probably is placed in or close to this region.

The assignment of primary sites to regions 2 and 3 is supported by studies showing separate binding sites of ligands. Brodersen et al. (32) have shown independent binding of bilirubin (region 3) and benzodiazepines (region 2). The independent binding of these compounds has also been suggested by Roosdorp et al. (222). Brodersen (28) found that the first molecule of acetyl-L-tryptophan (region 2) is bound to a locus other than the first site for bilirubin. Jacobsen and Jacobsen (107) observed that binding of L-tryptophan to albumin is unaffected by binding of the first molecule of bilirubin. The primary site of phenol red (region 3) is not placed in the same region as that of L-thyroxine (133), chloride ions (132), or octanoate (130), the primary sites of which are all placed in region 2.

Soltys and Hsia (249) have shown that the high affinity bilirubin binding site on human serum albumin has the capacity to bind 2 molecules of a monoanionic spin label with different affinities. The spin label studied was 1-γ-aminobutyrate-5-N-(1-oxyl-2,2,6,6-tetramethyl-4-aminopiperidinyl)-2,4-dinitrobenzene (GABA-DNB-SL) (see fig. 6). Interestingly, familiar spin labels possessing two carboxylic groups are bound to the bilirubin binding site according to a 1:1 model (96, 249). These observations point to the importance of two separate ionizable groups on ligands bound to that site.

Kragh-Hansen et al. (133) observed that addition of 1 mole of bilirubin to a complex of human albumin and phenol red resulted in a displacement of the dye in more than a stochiometric amount.

Flanagan and Ainsworth (68) studied the binding of trypan blue and 1-anilino-8-naphthalene sulphonic acid (ANS) (fig. 7) and suggested that the part(s) of bovine serum albumin associating 1 molecule of trypan blue could bind 2 ANS molecules.

Thus, it is possible that a binding region suited for combining 1 molecule of one type of ligands can bind 2 molecules of another ligand or perhaps two different, but probably smaller, ligands.

D. Binding Region 4

Most albumins are able to associate with positively charged inorganic ions in a reversible manner (table 4). Especially the combination with the first Cu^{++}, and apparently also Zn^{++} (198), is characterized by high binding constants. Binding of Cu^{++} (208, 20) and Ni^{++} (128, 206) and perhaps other metal ions occurs at a special and well-defined binding site composed of the first three amino acids from the amino terminal end of the albumin molecule (see section VIII B). Sarkar and co-workers (138, 139) studied the interaction between Zn^{++} and a tripeptide analogue of the N-terminal end of human serum albumin. The authors found that the N-terminal end of human albumin fulfills the conditions for being a well-defined location for tight binding of Zn^{++}. Analytical potentiometric studies (139) showed that the tripeptide bound Co^{++} less tightly than Zn^{++}. In contrast to the

above-mentioned ions, Ca^{++} and Mg^{++} apparently are bound to a common series of low affinity binding sites (201).

E. Binding Region 5

Haemin is bound to human albumin with a high association constant (table 1). Beaven et al. (10) concluded that binding of haemin to the primary site is not competitively influenced by oleate even at a very high fatty acid to protein ratio (50:1). In accordance with this observation Adams and Berman (1) found that addition of 10 moles of oleate per mole of human albumin did not displace haemin from its high affinity binding site. These findings indicate that identity of the primary binding sites of haemin and long-chain fatty acids (region 1) does not exist. Beaven et al. (10) concluded that the primary binding sites of bilirubin (region 3) and haemin are not identical. This point of view is in accordance with the proposal of Liem and Mueller-Eberhard (145). It seems unlikely that the primary haemin binding site is placed in region 2. According to the present binding model of albumin a secondary site of long-chain fatty acid ions (e.g. oleate) is placed in region 2. Explaining the observation of no haemin displacement from its primary binding site in the presence of 10 to 50 moles of oleate per mole of albumin by the difference in the association constants does not seem reasonable. Although the secondary association constant of oleate ($4.0 \times 10^6\ M^{-1}$) is lower than the primary association constant of haemin ($5–10 \times 10^7\ M^{-1}$), a considerable proportion of the bound haemin is expected to be displaced.

Identity of the primary sites of haemin and Cu^{++} ("region" 4) is also unlikely. Experiments with albumin fragments revealed that haemin is bound close to the middle of the albumin molecule, whereas Cu^{++} primarily is bound at the N-terminal end of the protein (see section VIII B). Thus, the primary haemin binding site is probably not placed in the regions 1 to 4. The binding site perhaps is identical to one of the (or the) secondary bilirubin binding site(s) (10).

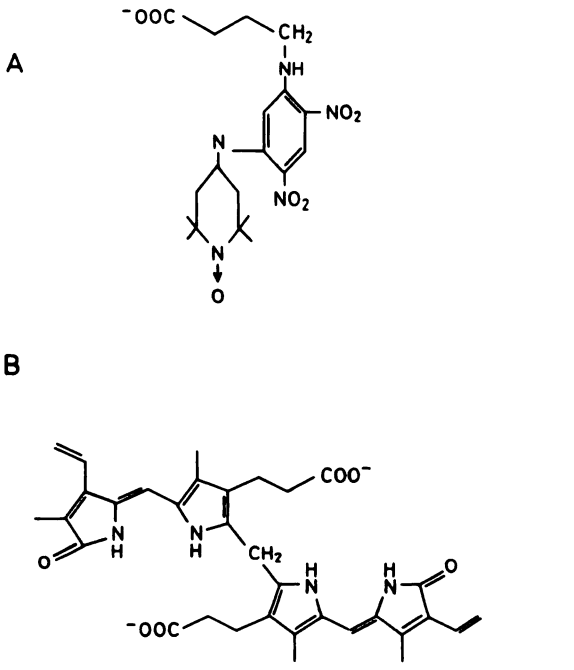


FIG. 6. Constitutional formulas: figure 6A, GABA-DNB-SL (1-γ-aminobutyrate-5-N(1-oxyl-2,2,6,6,-tetramethyl-4-amino-piperidinyl)-2,4-dinitrobenzene); figure 6B, bilirubin IX-α (z, z).

F. Other Binding Regions

It has often been suggested in the literature that a great number of drugs are bound to a common high affinity binding site of albumin. Among the drugs suggested are: warfarin (247, 248, 273), phenylbutazone (247, 248, 273), dicoumarol (204 49), tolbutamide (248, 37), and indomethacin (248). Seldom, however, have the suggestions been based on competitive binding studies, where the concentration of *free* inhibitor and the drug being inhibited has been determined. Furthermore, in some studies the molar ratio between ligand and albumin has been above unity. This implies that the competition phenomenon has not been studied at one binding site but at two or more sites. The binding to secondary sites is

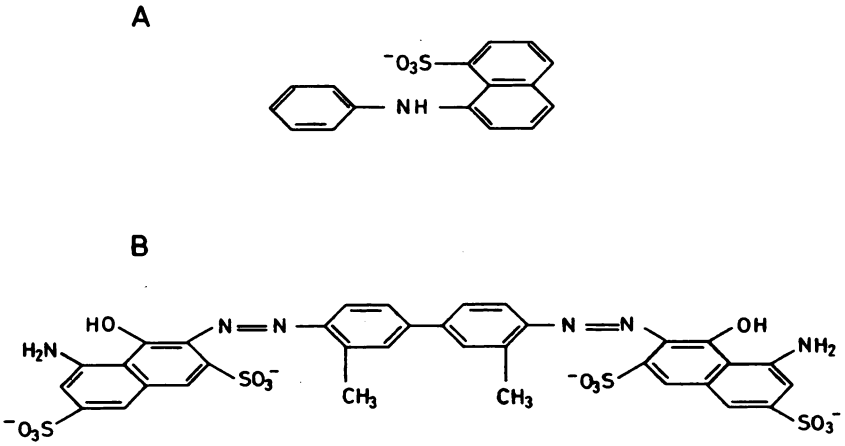


FIG. 7. Constitutional formulas: figure 7A, 1-anilino-8-naphthalene sulfonate; figure 7B, trypan blue.

very significant when the binding constants of the primary and secondary sites do not differ markedly or if the number of secondary sites is large.

Nevertheless it appears probable that binding regions other than those discussed in the previous sections exist for binding of various drugs on the albumin molecule. We shall examine in detail the evidence of a separate binding region for salicylate on albumin. The binding of this drug has been thoroughly studied (263, 28, 129, 224). Competitive binding studies by Tabachnick (263) probably indicate that only a secondary binding site for salicylate is in common with the high affinity thyroxine site (region 2). Brodersen (28) proposed that the high affinity binding site of bilirubin (region 3) and salicylate are not placed in the same region on human albumin. Kragh-Hansen (129) carried out competitive binding studies with low molar ratios of L-tryptophan, phenol red, and salicylate to human serum albumin. The binding data, which were analyzed according to equation 10 of the "Appendix," indicated that the primary binding site of salicylate is not in common to that of either tryptophan (region 2) or phenol red (region 3). Rudman et al. (224) observed that addition of 3.5 moles of palmitate to 1 mole of albumin did not result in a significant reduction in the association constant of salicylate binding to the primary site. This observation indicates that the high affinity binding site of salicylate is not placed in region 1 or 2. Addition of 7 moles of palmitate or oleate caused a very pronounced decrease of the first binding class (224), suggesting that the first site of the drug is not located in region 5. These observations seem to indicate that the primary site of salicylate is not located in the binding regions 1, 2, 3, or 5, and it is unlikely that the site is located in region 4.

Hultmark et al. (99) presented evidence for competitive binding of salicylate and indomethacin to a common primary binding site on human albumin.

Sebille et al. (236) have shown that the simultaneous binding of warfarin and furosemide to human albumin follows a competitive binding scheme. The relation between this common binding site and the high affinity site of salicylate and indomethacin awaits further clarification.

Chakrabarti (43) and Chakrabarti et al. (44) studied the binding of warfarin to human albumin in the presence of long-chain fatty acids. They observed that binding of warfarin was unaffected or somewhat increased in the presence of fatty acids in molar ratios to protein of 0 to 3. However, at ratios greater than 5 the binding constant of warfarin to the first site was decreased. Wilding et al. (291) also studied the influence of long-chain fatty acids on the binding of warfarin to fatty acid free human serum albumin. Binding of warfarin increased until about 3 moles of oleate or palmitate per mole of protein had been added. At higher fatty acid to albumin ratios the warfarin binding decreased and the binding percents in the absence of fatty acids were obtained in the presence of about 6 moles of oleate and about 8 moles of palmitate per mole of albumin, respectively. The influence of fatty acids on the warfarin binding could indicate that the drug is bound to the same region as bilirubin. However, as seen below, circular dichroic studies indicate that warfarin perhaps is bound to the same high affinity site as salicylate.

In some studies the free concentration of the ligand being inhibited has been determined by indirect means, e.g. as changes in fluorescence or ellipticities of the drug-protein complex. These methods do not take into account that the parameters measured could be changed by, e.g., conformational changes of the drug-protein complex introduced by binding of the second ligand to another region of albumin, or conversely, that the drug could be bound to another site giving the same fluorescence or ellipticity when the inhibitor is bound. The fact that the fluorescence or ellipticity decreases does not necessarily mean that the drug in question is unbound. It could be bound to a site on the protein not resulting in a detectable fluorescence or ellipticity. The possibility exists that competition may occur between a drug bound to a site not resulting in fluorescence or ellipticity and the inhibitor. Furthermore, the fluorescence could be reduced through energy transfer to the other ligand simultaneous bound.

Perrin and Nelson (203) published the results of a circular dichroic study of the competitive effect of various drugs on the binding of sulfaethidole to its high affinity site on bovine serum albumin. According to equilibrium dialysis the sulfonamide is bound to one primary site (association constant = 1.2×10^5 M^{-1}) and three secondary sites with a binding constant two orders of magnitude lower (1.0×10^3 M^{-1}) (203). Optical activity was registered only when the drug binds to the primary site so that binding to the secondary sites could be ignored in the circular dichroic studies. The authors suggest (203) that on bovine albumin sulfaethidole and sulfathiazole compete for the same primary site. This conclusion can be valid if possible effects of conformational changes are excluded. On the other hand the competitive effect of sulfadiazine and sulfacetamide was not significant, perhaps due to low binding constants of the drugs. But salicylate, chlorpropamide, tolbutamide, and perhaps warfarin seem to bind to the same site as sulfaethidole. Finally it was observed that pentobarbitone, thiopentone, and the positively charged drugs isopropamide iodide, edrophonium chloride, and pyridostigmine bromide do not compete for this site on bovine albumin.

In summary, a binding region seems to exist on albumin to which salicylate, indomethacin, sulfaethidole, sulfathiazole, chlorpropamide, tolbutamide, and perhaps warfarin are bound with a high affinity.

Mudge et al. (181) suggested that the primary binding sites of iopanoate and bromophenol blue are located in the same region, whereas the sites of iophenoxate and sulfobromophthalein are located in two other regions. As mentioned previously, the iopanoate-bromophenol blue site has been suggested to be located in region 3. How-

ever, the relation between the iophenoxate site and the sulfobromophthalein site and the other regions in the present proposal cannot be clarified at present.

To investigate the possible competitive effect of ligands bound simultaneously to albuin, it is necessary to operate with individual binding sites and not to conclude on the basis of changes in binding classes. With binding classes the freedom to pair the individual binding sites is reduced a priori. For example, the displacing effect of several drugs (salicylate, warfarin, phenylbutazone, sulfaphenazole, and paracetamol) on the binding of tolbutamide, chlorpropamide, and glibenclamide has been analyzed in this way (37). According to a competitive binding scheme the theoretical binding constants of the three drugs mentioned when bound to the first binding class in the presence of the inhibitor were calculated. The constants calculated were not in agreement with those determined experimentally and the conclusion of noncompetitive displacement was drawn. However, since the binding constants were not describing binding to individual high affinity sites, but were the average values of binding classes, which consisted of two to three sites, such a conclusion is invalid.

V. Binding of Steroids to Albumin

In blood plasma steroids are bound very tightly to globulins (association constants of the order 10^8 M^{-1}). Nevertheless at high concentrations, the steroids mainly associate with albumin. Due to the differences in the concentrations and binding affinities of the proteins, globulins have been termed low capacity-high intensity transport proteins and albumin a high capacity-low intensity transport protein.

Experiments for examining the association of a steroid to albumin and a possible competitive effect on the binding of other steroid- and non-steroid-ligands are troublesome due to the low solubility of most steroids in aqueous solutions. Nevertheless, Harding and Westphal (90) have been able to study the competitive binding of the chemically closely related steroids progesterone and deoxycorticosterone to human serum albumin with equilibrium dialysis. They reported that the two steroids share the same high affinity binding site. However, there are some reports in the literature that could indicate that testosterone and progesterone may be bound to different binding sites.

Ryan (226) studied the influence of several steroids on the tryptic digestion of albumin. He found that although the binding affinity of progesterone for human albumin is higher than that of testosterone (cf. table 1), its inhibitory effect on proteolysis is lower. Furthermore, he observed that the pattern of protein fragments in the presence of progesterone was different from that obtained in the presence of testosterone. Ryan and Chopra (227) observed that addition of 3 moles of palmitate per mole of protein results in a pronounced increase of the difference spectrum in the region 240 to 280 nm of testosterone-defatted human albumin, whereas the fatty acid had only a slight effect on the difference spectrum of progesterone-defatted human albumin.

Harding and Westphal (89) and Ryan and Chopra (227) studied the binding of progesterone (89, 227) and testosterone (227) to defatted albumin in the presence of fatty acids. According to Harding and Westphal (89), the binding of progesterone to delipidated human albumin is influenced by the presence of 5 moles of myristate. The number of binding sites (n) was reduced from four to two, whereas the association constant (K) was the same. Addition of 5 moles of laurate also caused a reduction of n. However, in this case a minor reduction of K was also observed. Thus, these authors observed a decrease in steroid binding in the presence of the fatty acids mentioned. Ryan and Chopra (227) found that addition of 3 moles of palmitate resulted in a reduction of n and an increase of K of progesterone and testosterone bound to bovine or human serum albumin. The effect observed was most pronounced in the case of bovine albumin. Addition of 5 moles of laurate to the bovine albumin-steroid complexes resulted in the same changes, although the influence was greater than that of palmitate. In all cases the resulting effect was an increased steroid binding (increased $n \times K$ values). This is in contrast to the findings of Harding and Westphal (89). Ryan and Chopra (227) suggested that the conflicting changes in $n \times K$, which mainly are caused by differences in calculated K values, might be due to a milder defatting procedure in their investigation. It is not possible, on the basis of the two studies cited, to determine the relation between the high affinity sites of progesterone, testosterone, and fatty acids.

Soltys and Hsia (250) determined the effects of nine different steroids on the binding of two structurally closely related spin labels, which selectively interact with the bilirubin binding sites (249). All experiments were carried out with a steroid-albumin molar ratio of 0.5. It was concluded that progesterone either competitively or more indirectly reduced the binding of spin label to the first binding site, whereas binding of spin label to secondary sites was enhanced. The overall effect was an increased spin-label binding as determined by electron spin resonance spectroscopy. The same observations were made in the presence of testosterone and estradiol. Although the effect of cortisone on spin-label binding was principally the same, cortisone displayed a net inhibition of binding at low spin label-albumin ratios (below 2) and an enhancement at higher ligand-protein ratios. The same dual influence was registered in the presence of hydrocortisone, deoxycorticosterone, corticosterone, estriol, and aldosterone. Soltys and Hsia (250) concluded that all the steroids mentioned can compete with the spin labels bound to the bilirubin binding sites.

Morrisett et al. (179) investigated the binding of spin-labelled stearate, palmitate, indole, and the steroid androstol to bovine albumin by electron spin resonance spectroscopy. They observed that although the spin-labelled steroid is bound with a lower affinity than the

fatty acids, the steroid is as strongly immobilized by albumin as the spin-labelled fatty acids. Chambaz et al. (45) and Basset et al. (9) studied the binding of other spin-labelled steroids to albumin and found the same immobilization of the bound ligands (comparable $2T_{11}$ values). These authors, however, proposed that the $2T_{11}$ values also can be interpreted as binding to sites with comparable hydrophobic features. Temperature studies showed (9) that bound spin-labelled dihydrotestosterone became more mobile and that the hydrophobicity of the binding sites of the steroid increased at higher temperatures.

In summary, the results reported in the literature indicate that progesterone and deoxycorticosterone are bound to the same site, whereas progesterone and testosterone perhaps are bound to different sites on albumin. Several steroids can bind to the same region on albumin as bilirubin, but the binding constants of these associations are not determined. Progesterone and testosterone interact with fatty acid binding sites. This interaction, if competitive, presumably takes place to secondary fatty acid sites, whereas an association between the primary sites of the two types of ligands is improbable.

VI. Specificity of Albumin Binding

Due to the large number of various ligands that can bind to albumin, the binding capability of the protein has often been regarded as nonspecific. In the following we have provided evidence against such a generalization. On the basis of the foregoing discussion it seems realistic to operate with at least six different binding regions. Regions 1, 4, and 5 seem to be very specific when compared with the other regions (cf. table 5). As already indicated, only long-chain fatty acids bind with a high affinity to region 1. Furthermore, long-chain aliphatic ligands with a charged group other than a carboxyl group—e.g. a sulfate or a sulfonate group—show different binding characteristics (213, 219). It has not been clarified whether these ligands can bind to this region at all. Regions 4 and 5 apparently are well suited for binding of metallic ions and haemin, respectively. Binding regions 3 and 6 are involved in the interaction with aromatic ligands of a diverse nature. When other types of ligands (aliphatic or inorganic) interact with these regions they do so with a low affinity. Apparently the least specific region is no. 2, which includes the primary sites of different aromatic, short-chain aliphatic, and inorganic ligands. In addition this region probably functions as a secondary binding site of long-chain fatty acids. Since the primary binding constant of octanoate [$8.3 \times 10^4\ M^{-1}$ (92, 130)] and the secondary binding constants of long-chain fatty acids (about $10^6\ M^{-1}$) do not largely differ, binding of fatty acids are characterized by affinities that are almost independent of chain length. Furthermore, the binding constants of the aromatic ligands bound with a high affinity are comparable to those of the aliphatic ligands [$10^4\ M^{-1}$ (tryptophan)–$10^6\ M^{-1}$ (tyroxine)]. However, the binding constant of the chloride ion is significantly lower (cf. table 4). Thus, the unique capability of albumin to bind ligands with a high affinity is based on the existence of several binding regions possessing various degrees of specificities.

Substitution of ligands with relatively simple groups can cause great changes in the ligand-protein interaction. For example, iopanoate and iophenoxate, which differ by an amino group in the structure of iopanoate and a hydroxyl group in the case of iophenoxate, are bound to different high affinity binding sites of human albumin (181). Furthermore, ampicillin, in contrast to penicillin G (they differ by an amino group), is able to displace bilirubin from human albumin (143). Benzoate displaces bilirubin from albumin, whereas addition of parahydroxybenzoate results in a stable ternary complex (143).

Ligand binding to albumin is in some cases stereospecific. D-Tryptophan is bound to human (164) and bovine albumin (120) with a binding constant about 100-fold *lower* than that of L-tryptophan. However, *d*-oxazepam hemisuccinate (a benzodiazepine) is bound to human albumin with a binding constant 30- to 40-fold *greater* than that of the *l*-isomer (184). The *d*-form of the ligand is also bound to bovine albumin in preference to the *l*-form (183).

In contrast to the above-mentioned ligands, the affinities of *d*- and *l*-warfarin for human albumin are very much alike (239). Furthermore, the *d*- and *l*-forms of progesterone and aldosterone bind equally well to human and bovine albumin, respectively (278). However, the binding constants of steroids are not the same whether substituents are located in the α- or β-position (288).

Isomeric compounds can interact with albumin in different ways. For example, Burkhard et al. (41) observed that ortho-aminobenzoate is bound to bovine albumin in preference to the meta-isomer, which is bound more strongly than the para-form. When the structurally related dye methyl red interacts with bovine albumin the binding preference is meta- > ortho- > paramethyl red (41). Binding studied at two different temperatures (0° and 25°C) indicated that the association of the meta-isomer, in contrast to the two other isomers, is temperature-dependent. Surprisingly, the three methyl red isomers apparently do not compete for the same primary binding site on albumin. Finally, addition of the aminobenzoate isomers can reverse the changes in the spectra of the ortho- and para-, but not the meta-form, of methyl red observed upon addition of albumin. In this example the greatest difference in binding seems to be between the meta-isomer and the two other forms.

Although albumin is able to bind a great number of different ligands, the binding constant and the binding specificity may be critically dependent on the presence of a particular chemical group.

VII. Examples of Covalent Binding to Albumin

The amino acid sequences of human (11, 168) and bovine (33) serum albumin indicate that both proteins contain a free sulfhydryl group in position 34. However,

only about 60% of the sulfhydryl groups in a solution of nondefatted albumin (98, 118, 3, 194) and 40% of defatted albumin (194) are reactive. Most of the unreactive sulfhydryl groups are blocked through covalent binding to cysteine or glutathione (118, 3). The residue of cysteine 34 also is able to form a disulfide bond with other substances, e.g. lysine vasopressin (63). Furthermore, the cations Ag^{+} and Hg^{++} probably are able to interact covalently with this amino acid residue of albumin (206).

A very interesting example of covalent binding to albumin is that of D-glucose (58, 61, 87). Simple incubation in vitro of human albumin with glucose in the millimolar range for hours or days resulted in glycosylation of the protein. Glucose probably reacts with the amino groups of lysine residues. According to Day et al. (58) glucosylated albumin accounted for 6% to 15% of total albumin in serum from healthy donors. Dolhofer and Wieland (61) also found that glucosylated albumin is a natural constituent of normal serum. Furthermore, they and Guthrow et al. (87) observed that this albumin fraction is increased by a factor of 2 to 3 in serum from diabetic persons. Measurements of glucosylated albumin may in future be used as an indicator of the status of diabetic persons and to detect cases of subclinical diabetes (87).

Recent studies by Maclouf et al. (152) indicate that prostaglandin H_2 and thromboxane A_2 (a mediator of irreversible platelet aggregation) also bind covalently to human serum albumin.

It is noteworthy that the commonly used drug acetylsalicylic acid (aspirin) is covalently bound to a lysine residue of human serum albumin (91)—probably placed in position 199 (281). Furthermore, Bundgaard (38) reported that several penicillins (benzylpenicillin, phenoxymethylpenicillin, ampicillin, carbenicillin, and cloxacillin) acetylate ϵ-amino groups of lysine residues and imidazole groups of histidine residues of human and bovine serum albumin by reaction with the β-lactam ring of the penicillins.

According to Hilak et al. (94), the primary site of pyridoxal 5′-phosphate is located in the N-terminal half of bovine albumin. The ligand forms a Schiff's base with the amino group of a lysine residue, probably no. 223 (2, 207). Moreover, fluorescein sensitized by photooxidation binds covalently to tyrosine 137 of bovine albumin (23, 25).

Peroxidized phospholipid (190, 192) and other peroxidized lipids (268) are bound covalently to human albumin. The reaction is probably unspecific, since binding to γ-globulin occurs to the same extent (191). The reaction, which causes major changes in the structure of albumin (192, 268), was studied as a model for deteriorating effects of peroxidized lipids on proteins (including membrane proteins) in vivo.

The ability of albumin to interact covalently with several different chemical substances has been widely used in labelling amino acid residues of the protein. If modification of an amino acid residue results in a pronounced reduction of ligand binding, it is usually concluded that the residue modified is placed in or close to the binding site of the ligand investigated. However, such a conclusion can be drawn only if conformational changes of the protein are excluded. Reviews on chemical modifications of albumin and other proteins have previously appeared (93, 166). Ligand binding to modified albumins will be further dealt with in the following section of this review.

VIII. Ligand Binding Locations of Albumin

A. Ligand Binding to Chemically Modified Albumins

How are the binding regions of albumin constructed? An important aspect of this problem is an identification of amino acid residues lining the binding region. A commonly used way of approaching this problem is to modify specifically amino acid residues of the protein by reactive compounds and afterwards to measure the ligand binding ability of the modified albumin molecule, as compared with that of the native protein. Such studies can be supplemented by a sequence analysis determining in which position of the albumin sequence the covalently attached modifier is placed. Two major difficulties inherent in this approach should be mentioned. Firstly, the reagent is seldom specific, i.e. more than one kind of amino acid residues is usually modified. The specificity can be improved by a careful selection of experimental conditions such as pH, reaction time, and molar ratio between reagent and protein. Secondly, often conformational changes of albumin are introduced during the labelling procedure. Therefore, the mildest possible conditions should be selected during this procedure, with concurrent control of protein conformation. It should be emphasized that all studies cited in this section have concentrated on an attempt to identify the kind of ionizable amino acid residues involved in the binding of the ligand in question. Furthermore, the great majority of the investigations have focused on the combination of negatively charged ligand and positively charged amino acid residues. A limitation is that specific labelling of the hydrophobic amino acid residues of albumin (except for tryptophanyl and cysteinyl) has not been achieved. However, if the position of the ionizable residues can be determined, knowledge of the sequence of albumin is a great help in a judgment of the identities and positions of the hydrophobic residues assisting in forming the binding regions.

Another possibility of estimating the amino acid residues lining the binding regions is to determine in which position of the albumin molecule reactive ligands are covalently bound. In this section a brief review of the studies of ligand binding to modified albumins is given. The following account is based on the binding regions proposed in table 5.

The literature dealing with binding of long chain fatty acids (binding region 1) to modified albumin is scarce. The only information provided by such kind of studies is that a fairly reactive amino group of a lysine residue probably is part of the region (4, 85, 253).

Binding of several of the ligands, with primary binding site in region 2, to modified albumins has been studied. Tritsch and Tritsch (274) investigated the interaction of L-thyroxine and albumin modified extensively with difluorodinitrophenyl sulfone, *O*-methylisourea, acetic anhydride, or ninhydrin. All reagents react primarily with amino groups on the protein, and the authors found that the binding ability of all modifications was greatly reduced, except for the guanidinated albumin, which could bind thyroxine with almost the same affinity as the unmodified protein. The authors suggested that the α-amino group of albumin is of primary importance in thyroxine binding. They observed that thyroxine binding to human and bovine serum albumins, at pH 7.35, was indistinguishable. The authors considered that the fact that the proteins have the same amino-terminal amino acid (aspartic acid), whereas the overall amino acid compositions are different, lent support to the above-mentioned suggestion. Furthermore, the authors probably were guided by the idea that the α-amino groups could be more reactive than the ϵ-amino groups of the two proteins. More recent studies have not supported the idea proposed. Tritsch and Tritsch themselves have presented results indicating that thyroxine and tryptophan bind to the same high affinity binding site (274). A great number of studies (see below and section VIII B) indicate that this common binding site is placed close to the middle of the albumin molecule and not at the N-terminal end. Other studies have shown that albumin possesses at least one highly reactive ϵ-amino group (79, 80).

Tabachnick (262) also has studied the binding of L-thyroxine to extensively modified albumins. Treating human serum albumin with acetic anhydride resulted in a pronounced reduction of L-thyroxine binding, whereas the binding was not reduced after extensive guanidination. Thus, the two investigations (274, 262) agree that blocking of free amino groups by nonionizable labels reduces the binding. By contrast, replacing the amino group with another ionizable, basic group apparently does not reduce the binding significantly.

Binding of L-tryptophan to native and modified albumin preparations has been studied in several laboratories. McMenamy and Oncley (164) investigated the binding at pH 7.95 and found that acetylation of only 7% to 8% of the free amino groups of human serum albumin by acetic anhydride resulted in a very pronounced decrease in binding; $n \times K$ of binding to the protein was 4.95×10^4 M^{-1} and 0.87×10^4 M^{-1} before and after acetylation, respectively. Modification of 36% to 37% of the amino groups almost abolished binding. On the other hand, guanidination of 65% to 70% of the amino groups did not result in an appreciable alteration in the binding of tryptophan.

Jacobsen and Jacobsen (107) observed that dansylation of less than one amino group (a lysine residue) per human albumin molecule results in a decrease in the binding of L-tryptophan. Gambhir et al. (76) intensively investigated the binding of acetyl-L-tryptophan to a large fragment of human serum albumin, containing the indol binding site, modified by bromoacetyl-L-tryptophan, dansyl chloride or pyridoxal 5′-phosphate (ratio between modifying agent and protein was 1:1). The labels react with imidazole groups, amino groups of lysine residues plus hydroxyl groups from tyrosine residues, and amino groups of lysine residues, respectively (76). The authors concluded that the histidine residue in position 146 (numbered according to figs. 1 and 2) is essential for binding of indole compounds. Furthermore, binding of acetyl-L-tryptophan to the fragment modified by dansyl chloride or pyridoxal 5′-phosphate indicated that probably also a lysine residue is placed at the binding site. The authors found a small peptide, suggested to include only one lysine residue (no. 195 in the complete albumin sequence), to which a relative great percentage of the modificators could be found again and therefore could be a part of the indole binding site, since binding of acetyl-L-tryptophan was greatly reduced by the two modificators. However, in contrast to Gambhir et al. (76), Meloun et al. (168), and Brown (35) agree that this small part of human albumin contains not one but two lysine residues (no. 190 and 195). Thus, the observation, leading to the conclusion that lysine 195 is close to the binding site, may be explained by the existence of another lysine residue (no. 190) close to that in position 195, also capable of binding the modifiers. Other binding results from the same laboratory (75) with human serum albumin treated with dansyl glycine indicate that also a tyrosine residue, from another part of albumin than that studied above (76), is included in the binding site of acetyl-L-tryptophan. Furthermore, the importance of the two arginine residues adjoining histidine 146 has been stressed by McMenamy and coworkers (163, 76).

Fehske et al. (66, 67) studied the capability of native albumin and the protein modified by 2-hydroxy-5-nitrobenzyl bromide (66), *O*-nitrophenylsulphenyl chloride (67), or tetranitromethane (67) to bind L-tryptophan and four different benzodiazepines (diazepam, chlordiazepoxide, tetrazepam, and oxazepam), which probably are bound to the same site on albumin. Treating human serum albumin with 2-hydroxy-5-nitrobenzyl bromide at low pH selectively modified tryptophan 214 and resulted in a decrease of the association constants of L-tryptophan and the benzodiazepines by 30% to 50% and 10% to 15%, respectively. The number of binding sites was unchanged. The authors suggested that the tryptophan residue is not directly involved in the binding of the reversibly associated ligands. The reduction in binding constants was

suggested to be caused by indirect means, e.g. conformational changes (66). The same conclusion was drawn on the basis of results, where ligands were added to an albumin preparation with the tryptophan residue modified by *O*-nitrophenylsulphenyl chloride (67). Fehske et al. (67) observed that only 9 of the 18 tyrosine residues of human serum albumin could be modified by tetranitromethane. Modification of only 2.2 tyrosine residues per protein molecule resulted in a reduction of the association constants of L-tryptophan and diazepam by more than 90%. Furthermore, the extrinsic Cotton effects caused by binding of the four benzodiazepines to albumin were greatly reduced by the modification. Further studies at low tetranitromethane:albumin ratios revealed that probably only one tyrosine residue, which perhaps was more reactive than the other tyrosine residues, is placed in the part of albumin associating tryptophan and benzodiazepines. The authors suggest that the tyrosine residue could be one of the four residues close to histidine 146: 138, 140, 148, or 150. In this connection it is of great interest to note that Moravek et al. (178) observed that no. 148 and no. 150, in contrast to the other two tyrosines, are readily titratable by tetranitromethane.

Means and Bender (165) and Means and Wu (167) observed that a tyrosine residue of human serum albumin reacted rapidly with *p*-nitrophenyl acetate (165) and diisopropylfluorophosphate (167). The residue seems to have a relatively low apparent pK_a: 8.7 (165) or 8.3 (167). These authors and Koh and Means (126) proposed that the strongly reactive tyrosine residue is located in the high affinity binding site of fatty acids that contain 10 or less carbon atoms. Furthermore, Sollenne and Means (246) studied the inhibition of the acetylation of human albumin by various drugs. They found that the fast reacting amino acid residue probably is placed not only in the primary site of short-chain fatty acids but also in that of the following drugs: naproxen, ibuprofen, chlorophenoxyisobutyric acid, and chlordiazepoxide [in accordance with Fehske et al. (67)]. By contrast, the first site of indomethacin is placed elsewhere on the albumin molecule.

In summary, the following kinds of amino acid residues seem to take part in forming the binding region 2 of albumin: tyrosine (75, 67, 126, 165, 167), arginine (163, 76), and histidine (76) but not tryptophan (66, 67). The amino acid residues suggested seem to be placed mainly at the "top" of the long loop in subdomain 1C (cf. fig. 4): histidine 146, one or both of the arginines adjoining the histidine 146 and probably tyrosine 148 or 150. Lysine 195 (and 190) is placed in "helix" z of subdomain 1C. Arranging this "helix" according to figure 5 in reference 36 brings lysine 195 rather close to the other residues mentioned.

Among the ligands bound primarily to region 3 the association of bromophenol blue and bilirubin to chemically modified human serum albumin has been investigated. Binding of bromophenol blue to native albumin and to 14 different albumin preparations has been studied by Lind et al. (148). Mainly amino groups were modified and the authors made the following observations: 1) Extensive blockade of amino groups by other basic, ionizable groups (treatment with methyl acetimidiate or *O*-methylisourea) did not result in a detectable alteration of the association constants as compared with native albumin. 2) Small, nonionizable substituents like acetyl, carboxymethyl, and glyoxyl groups reduced dye binding affinity almost linearly with the number of substituted groups irrespectively of the kind of modification. 3) Aromatic, nonionizable substituents (dinitrophenyl and trinitrophenyl groups) caused a greater reduction of bromophenol blue binding per modified amino group. In all the modifications more than one residue of albumin was labelled (approximately 1.4 to 65 residues). It was concluded that bromophenol blue is bound to a part of albumin with a preponderance of positive charges and that the existence of the charges is more essential than the structure of the amino acids possessing them (lysine, arginine, or histidine) (148).

Jacobsen (103–106) investigated the binding of bilirubin to unmodified and several modifications of human serum albumin in an attempt to identify the amino acid residues involved in the bilirubin binding site. As in the case of bromophenol blue, extensive modification with methyl acetimidiate did not result in a decreased ligand binding (103). Furthermore, treatment with acetylsalicylic acid, ethyl diazoacetate, and *O*-nitrophenylsulfenyl chloride resulted in none or only minor changes in bilirubin binding, indicating that neither lysine 199 (281), carboxyl groups, the tryptophan residue in position 214, nor the cysteine residue in position 34 is essential for binding. The reduced binding of albumin treated with succinic anhydride, maleic anhydride, N-acetylimidazole, acetic anhydride, tetranitromethane, diethylpyrocarbonate, or glyoxal (103) often was accompanied by conformational alterations of the protein as revealed by changes in Stokes radius and electrophoretic mobility. In all the albumin preparations studied one or more residues had been labelled. Although conformational changes seldom could be excluded, it was suggested (103) that the following kind of amino acid residues were close to or located in the high affinity bilirubin binding site: histidine, arginine, and tyrosine.

In a later publication (104) Jacobsen reported the results on the binding of bilirubin to albumin treated with trinitrobenzenesulphonic acid and picrylchloride at low reagent to protein ratios. The study also includes binding experiments with albumin preparations in which the binding site was occupied by bilirubin during the modification procedure. The results also indicated that one or two ϵ-amino groups of lysine are essential for the high affinity binding of bilirubin (104). Recently, Jacobsen and Jacobsen (107) observed that the first lysine residue of human albumin blocked by dansylation at low reagent:protein ratios is not associated with binding of

bilirubin. According to Gambhir et al. (76) this lysine residue could be placed in position 195. Thus, neither lysine 199 nor lysine 195(?) is essential for the high affinity binding of bilirubin (107). However, dansylation of two or more lysine residues decreased binding of bilirubin.

Jacobsen suggested, on the basis of experiments with bilirubin covalently bound to albumin, that the lysine residue placed in the high affinity site of the ligand is no. 240 (105, 106). Coupling of bilirubin to albumin with carbodiimide as a catalyst was followed by tryptic fragmentation of the protein. A peptide consisting of 19 amino acid residues, to which most of the bilirubin was bound, was isolated, and the amino acid composition was determined. No sequence analysis was carried out but the composition was comparable to that of the sequence 240 to 258 of human albumin. This sequence contains only one lysine residue, namely in position 240.

In summary, the results cited above propose that in binding region 3 a preponderance of basic amino acid residues (lysine 240, arginine, and histidine) and possibly tyrosine exist.

As far as the author is aware, neither binding of Cu^{++} nor Ni^{++} (region 4), haemin (region 5), nor the drugs assigned to region 6 (cf. table 5) to modified albumin have been investigated.

B. Ligand Binding to Serum Albumin Fragments

At which positions in the albumin molecule are ligands bound with a high affinity? One approach to that problem, as discussed in the previous section, would be to determine one or more of the amino acid residues lining the binding site and to correlate the findings with the amino acid sequence of albumin. An alternative possibility would be to study ligand binding to fragments of albumin. According to this procedure a part of albumin is isolated, which is able to bind the ligand in question with the same, or slightly lower, affinity as that of the intact protein. The position in the albumin sequence of the fragment is determined by comparing the amino acid composition or, preferably, sequence of the fragment with appropriate sections of the known, complete amino acid sequence of albumin.

Fragmentation of albumin by enzymes and cyanogen bromide gives fragments with intact disulfide bonds, and many possess the ability to bind various ligands. Large fragments as a rule contain the same or a slightly less amount of α-helix as the native protein according to circular dichroic measurements, whereas the content of β-structure of the fragments varies as compared with that of native albumin (244, 78, 216, 242, 95).

Peters and coworkers (209, 216) studied the binding of palmitate (binding region 1) to fragments of bovine serum albumin. Peters and Feldhoff (209) bound albumin to palmitate immobilized on agarose. Afterwards the bound protein was digested by trypsin and the fragment still bound to the palmitoyl-agarose column consisted of domain 3 (loops 7 to 9). This indicates the existence of a strong binding site in this domain (209). Reed et al. (216) studied the binding of palmitate to fragments obtained by tryptic proteolysis of albumin in solution. These studies indicated that the primary site of the fatty acid is placed in subdomain 3A-B (sequence 377 to 503). [More recently Peters and Reed (210) assigned the primary palmitate binding site to loop 7 (ca. 390–450)]. Furthermore, the second and third binding sites were assigned to the sequences 239–306 and 307–377, respectively. Andersson et al. (4) found that trinitrobenzenesulfonic acid binds primarily to a part of bovine albumin with the sequence Leu-Ala-Glu-Lys-Tyr. Palmitate competes strongly with the labelling of this part of albumin. In bovine albumin only the sequence of one section resembles that of the pentapeptide mentioned: 347–351 (Leu-Ala-Lys-Glu-Tyr). This finding indicates that in the fragment 307–377 the residues 347–351 could be the most essential for fatty acid binding.

Binding of ligands with a high affinity binding site in region 2 to albumin fragments also has been studied. Sjöholm and Ljungstedt (244) investigated the binding of fragments of human serum albumin, obtained by cyanogen bromide digestion, to L-tryptophan linked to a Sepharose column. The only fragment bound to the immobilized amino acid was a fragment C consisting of 157 amino acids including the lone tryptophan residue (position 214). King and Spencer (120) split bovine albumin by trypsin into two large fragments consisting of approximately the amino terminal one third and of the carboxyl terminal two thirds of the protein. L-Tryptophan binds to a site of the latter fragment with a binding constant about half of that found for the association of the ligand to native bovine albumin. When bovine serum albumin was digested with the aid of pepsin into two big fragments, corresponding roughly to the N- and C-terminal half of the protein, King (119) observed that only the C-terminal part bound L-tryptophan.

Geisow and Beaven (78) produced three large fragments of human serum albumin by peptic digestion, namely P44 (residues 1–386), P29 (49–307) and P31 (308–585). P44 contains all the strong binding sites of 1-anilino-8-naphthalene sulfonate. In contrast to P44 and P29, P31 was not able to bind the ligand firmly. Addition of acetyl-L-tryptophan to complexes of P44 or P29 and the fluorescent ligand indicated that the strong binding site of acetyl-L-tryptophan could be found in P44 but not in P29.

King and Spencer (120) and King (119) found that octanoate binds to the same fragments of bovine albumin as tryptophan.

Sjödin et al. (242) digested human serum albumin with trypsins by the procedure of King and Spencer (120), and obtained two fragments consisting of the sequences 1–181 and 182–585. According to circular dichroism studies the high affinity binding site of diazepam is retained in the larger fragment.

The primary binding sites of the ligands from region 2 mentioned in the foregoing are placed in the C-terminal two-thirds (182–585 of the human species) of albumin (119, 120, 242). According to Geisow and Beaven (78) the high affinity binding site of acetyl-L-tryptophan, which is assumed to be the same as that of L-tryptophan, is contained in a fragment consisting of residues 1–386. Thus, the attention is focused on residues 182–386. The results of King's (119) study indicating that the binding sites of octanoate and tryptophan are placed in the C-terminal half of bovine albumin [residues 306–582 (92)] probably reduce the range of essential residues to 306–386. This sequence is surprisingly far from the N-terminal end as compared with the results obtained by binding to chemically modified albumins.

The only representative of ligands of region 3 with which binding experiments with albumin fragments have been carried out is bilirubin. Sjödin et al. (242), with circular dichroism, found that the primary site of this ligand is contained in a fragment composed of residues 182–585. Geisow and Beaven (78), also with circular dichroism in the study of ligand binding to albumin fragments, observed that bilirubin interacts strongly with the fragments P44 (1–386) and P29 (49–307) but not with P31 (308–585). Reed et al. (216) suggested that the primary site of bilirubin is associated with the residues 186–238 of bovine albumin. Kuenzle et al. (136) covalently labelled the bilirubin binding sites of human serum albumin by bilirubin activated with Woodward's reagent K. The same authors (81) mentioned that a subsequent fragmentation of the labelled protein by cyanogen bromide showed that the label was recovered in two peptides, residues 124–297 and 446–547. Reed (215) labelled bovine albumin with the same reagent as used by Kuenzle et al. (136) and subsequent isolation of fragments supported the proposal that the high affinity site of bilirubin is placed in the sequence ca. 198–251 (loop 4). Involvement of the residues from about 180 to 250 seems to be a reasonable conclusion from the five studies cited.

Binding of Cu^{++} and Ni^{++} ("region" 4) to fragments of albumin and to albumin from different species has been studied intensively and the results published before about 1975 have been summarized by Peters (206, 207). The ions form a chelate complex with the three N-terminal amino acids: Asp-Ala-His in human and Asp-Thr-His in bovine serum albumin (cf. figs. 1, 2, and 3). The nitrogen atom of the α-amino group of aspartic acid, the two nitrogen atoms in the peptide chain, one of the nitrogens (position 3) in the imidazole group of histidine, and the ion bound are the atoms most involved in forming the complex. Binding experiments with albumin from different species and synthetic peptides have shown that the amino acids in positions 1 and 2 can be exchanged with other amino acids, whereas substitution of histidine abolishes the binding (cf. dog albumin, section III E), indicating that histidine in position 3 is the key feature. Sarkar and coworkers carried out detailed investigations on the interaction of various metal ions and a tripeptide analogue of the N-terminal end of human serum albumin. Recently, Laussac and Sarkar (141, 142) published ^{13}C- and ^{1}H-nuclear magnetic resonance spectroscopy studies on the binding of Cu^{++} and Ni^{++} to the synthetic tripeptide. They found direct involvement of not only the four nitrogen atoms mentioned above but also of the carboxyl group of the aspartic acid residue. Lakusta and Sarkar (139) and Lakusta et al. (138) observed, with the same tripeptide as above, that a Zn^{++} binding site could be placed at the primary Cu^{++} and Ni^{++} binding site of albumin. Points of strong interaction for Zn^{++} would be the imidazole nitrogen of the histidine residue and the carboxyl group of the aspartic acid residue (rather than the amino terminal of the residue). The remaining coordination sites of the zinc ion could be occupied by solvent molecules.

Hrkal et al. (95) investigated the interaction between haemin (binding region 5) and three different fragments of human serum albumin obtained by cyanogen bromide treatment. Although all of the three peptides (1–123, 124–298 and 299–585) were able to bind haemin with a relatively high affinity, the magnitude of the binding constants and kinetic studies suggested that the primary binding site is located in the sequence 124–298. The authors proposed that the presence of the C-terminal part of albumin is essential for the spatial configuration of the haemin binding site.

Wilding et al. (291) studied the binding of warfarin to two big fragments of bovine albumin consisting of domain one plus subdomain 2A-B and subdomain 2C plus domain three (cf. fig. 4), respectively. The percent of binding of warfarin to each of the two fragments was not much different and was only about one-tenth of that determined for binding to intact albumin. A one-to-one combination of the two fragments increased the binding but could not fully restore the drug binding. It is tempting to suggest that the warfarin binding site is placed close to the middle of the albumin molecule, and that the native conformation is required for the formation of the high affinity binding site.

Reed et al. (216) reported on binding of 1-anilino-8-naphthalene sulfonate and bromocresol green to bovine serum albumin and 11 different fragments thereof. The binding strength of the ligands to the various fragments was roughly in proportion to the size of the peptides. However, some fragments exhibited appreciably higher affinities than the rest. A fragment consisting of loop 3 (subdomain 1C) bound 1-anilino-8-naphthalene sulfonate with a significant higher affinity, and fragments including loops 4 and 5 (subdomain 2A-B) interacted strongly with bromocresol green.

C. Summary of Ligand Binding Locations of Albumin

In this section the present information of ligand binding locations of albumin and the positions of the lone sulfhydryl group and the tryptophan residue (of human

albumin) is compiled (fig. 8). Furthermore, the results of studies estimating distances between some important locations on human albumin are given (table 6).

It is very interesting to note that a relatively great number of ligand binding sites seems to be placed in the part of the albumin molecule named subdomains 1C and 2A-B.

Binding experiments with chemically modified albumins propose that binding region 2 mainly is associated to the "top" of loop 3 (subdomain 1C). However, investigations of binding to fragments assign the region to domain 2. Both conclusions are based on several independent studies but are difficult to bring in accordance with the commonly accepted model of albumin consisting of three separate domains composed as shown in figure 4. The observations may be explained by assuming a rather close contact between subdomain 1C and parts of domain 2 (e.g. subdomain 2A-B).

According to Jacobsen (106), lysine 240 should be located in the primary site of bilirubin. Binding studies with fragments showed that this site probably is found in the sequence 180–250. Thus, these findings also suggest an association of the subdomains 1C and 2A-B. Berde et al. (14) and Chen (46) estimated the distance between the tryptophan residue and the primary bilirubin site of human albumin to be 27 to 28 Å (table 6). This information probably places the binding site in the "lower end" of subdomains 1C and 2A-B (fig. 8).

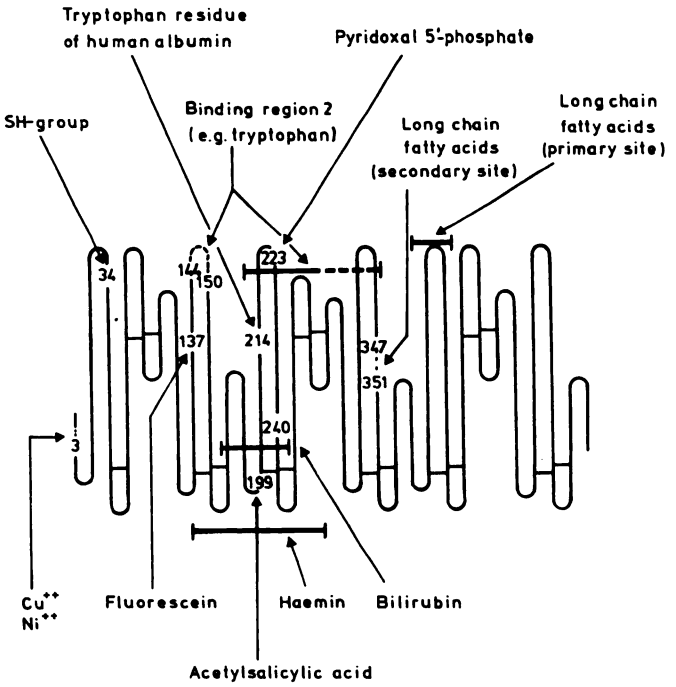


FIG. 8. Model of serum albumin showing the position of the lone cysteine residue, which is the same in bovine and human albumin (cf. figs. 1 to 3), and that of the lone tryptophan residue of human albumin. Furthermore, the positions proposed for several binding sites and regions are indicated. (For further details, see the text, sections VII and VIII.)

TABLE 6

*Distances between various locations of human serum albumin**

Location 1	Location 2	Distance, Å	Reference
Thyroxine (1)†	Tryptophanyl‡	22	202
Bilirubin (1)	Tryptophanyl	27	14
Bilirubin (1)	Tryptophanyl	28	46
Bilirubin (2)	Tryptophanyl	24	14
SL-parinaric acid (1)§	Bilirubin (1)	40	14
SL-parinaric acid (1)	Bilirubin (2)	37	14
SL-parinaric acid (2)	Bilirubin (1)	40	14
SL-parinaric acid (2)	Bilirubin (2)	35	14
Warfarin (1)	Tryptophanyl	34.5	49
Dansylglycine (1)	Tryptophanyl	23.7	49

* Distances on the albumin molecule calculated on the basis of energy transfer according to the mechanism described by Förster (69). According to hydrodynamic studies albumin is an ellipsoid with major and minor axes of 140 and 40 Å, respectively (206).

† The figures in the parenthesis denote a primary and secondary binding site, respectively.

‡ The tryptophan residue of human albumin.

§ SL, spin-labelled.

A contact between subdomains 1C and 2A-B has actually been proposed in alternative albumin models. Brown (35) has put forward an alternative 4-unit model in which subdomains 1A-B and 3C form two lateral, small units (no. 1 and 4) and subdomains 1C + 2A-B and 2C + 3A-B constitute two central, large units (no. 2 and 3).

Thus, the information especially from binding studies of ligands bound with a high affinity to regions 2 and 3 seems to be more in accordance with the 4-domain than the 3-domain model of albumin suggested by Brown (35). It should be noted that the "intradomainal" hinge regions of figure 4 (equals the peptides connecting the domains in the 4-domain model) are relatively long, polar, and nonhelical (see section III D). This observation agrees well with the 4-domain model.

IX. On the Flexibility of Albumin

A. Conformational Fluctuations of Albumin and Other Proteins in Aqueous Solutions

Proteins in solution are not static structures but molecules changing between different conformational states. This is revealed by, e.g., the ability of hydrogen atoms in the peptide bonds of proteins to exchange with deuterium (or tritium) in an aqueous solution (for reviews, see ref. 100 and 292). The isotope exchange can be measured by several techniques: infrared (100) and ultraviolet spectroscopy (65), a tritium-Sephadex method (64), and nuclear magnetic resonance spectroscopy (100, 170, 17). Benson et al. (13) studied the hydrogen-deuterium exchange of bovine serum albumin at different pH values (in the range of 3 to 8.5), by infrared spectroscopy. They observed four different classes of hydrogen atoms in the protein: one with atoms exchanging so fast that it could not be followed by the technique, two slow classes with

first-order rate constants of about 1×10^{-3} sec^{-1} and 3×10^{-5} sec^{-1}, respectively, and one class with very slowly exchangeable atoms (not exchanged after 24 hours at 25° C). No relationship was found between the α-helix content of the protein as determined by optical rotatory dispersion measurements and the hydrogen exchange parameters. The number of nonexchanged hydrogen atoms was greatest at pH 5. In a later publication (12) it was shown that this number was constant in the pH region 5.0 to 6.4. These observations probably indicate that albumin is most compact and stable in that pH interval. Interestingly, the isoionic point of albumin is placed in that pH region (267). At higher and lower pH values the reduction of the number of very slowly exchangeable hydrogen atoms was accompanied by an increase in the number of very fast exchangeable atoms. In the more recent publication Benson and Hallaway (12) have extended the hydrogen-deuterium exchange studies of bovine albumin and concluded that this protein has a high degree of motility brought about by noncooperative segmental movements. Hvidt and Wallevik (101) carried out hydrogen-deuterium studies of human serum albumin. They observed that also this protein has a high degree of motility with the greatest number of nonexchanged hydrogen atoms in the same pH-range as that of bovine albumin (fig. 2a in ref. 101). These authors also suggested that the exchange of hydrogen atoms of albumin proceeds as independent reactions (noncooperativity) of peptide groups. The rate constants of the various hydrogen atoms were so dispersed that these authors did not operate with classes of hydrogen atoms but preferred to represent the data in a form analogous to titration curves.

The rates at which the hydrogen atoms exchange are dependent upon the location in the protein molecule. It is generally agreed that hydrogens on the protein surface exchange readily with deuterium in the solvent, whereas hydrogens buried in the protein molecule cannot exchange unless contact with the solvent is established. Such contact is considered to be brought about by relatively large-amplitude fluctuations ("breathing") of parts of the protein. However, as indicated by the presence of nonexchanged hydrogen atoms, the breathing mechanism, under nondenaturating conditions, does not result in exposure of all parts of, e.g., serum albumin.

Other techniques, especially fluorescence techniques, have shown that small-amplitude fluctuations, probably in the form of vibrational and rotational motions, also occur in albumin.

Lakowicz and Weber (137) studied the quenching effect of molecular oxygen at high pressures on the fluorescence of aromatic amino acid residues in several proteins, including bovine and human serum albumin. These authors found evidence for the existence of structural fluctuations in the proteins on a nanosecond time scale. The fluctuations most probably involve separation of groups held together by energies not greater than a few kilocalories/mole (284). The rates of the fluctuations found experimentally are in accordance with those estimated by energetic calculations (285). Furthermore, Brand and Gohlke (22) carried out fluorescence lifetime measurements of the fluorescence of 2-*p*-toluidinylnaphthalene-6-sulfonate bound to bovine serum albumin or dissolved in organic solvents. The shifts observed under these conditions and at different temperatures are in accordance with the idea of nanosecond motions of polar groups at the dye binding sites of albumin (22). Munro et al. (185) studied the dynamics of human serum albumin and other proteins with a single tryptophan residue, by time-resolved fluorescence polarization spectroscopy. They observed that at 8° C the amino acid residue rotated together with the whole protein or a large part of it (rotational correlation time = 31.4 nsec), whereas at 43° C the tryptophan residue acquires rotational freedom independent of the whole protein and rotates very fast (rotational correlation time = 0.14 nsec).

Could the ability of albumin to change conformation, as indicated by the large- and small-amplitude motions, be an important factor for the pronounced capability to bind ligands with a high affinity? The ligand-binding ability of albumin is superior to that of, for example, ovalbumin and β-lactoglobulin, which have isoionic points close to that of serum albumin (131). In a review, Willumsen (292) compared the hydrogen isotope exchange of several proteins. He found that serum albumin is the peptide that most readily exchanges hydrogen atoms of the nonenzymatic proteins surveyed (except the small protein insulin): bovine serum albumin, light meromyosin, apomyoglobin, heavy meromyosin, plakalbumin, myosin, ovalbumin, and metmyoglobin (mentioned according to increasing stability). It is relevant to note that apomyoglobin exchanges more readily than metmyoglobin. Serum albumin also fluctuates more than β-lactoglobulin: After 200 min about 56% to 66% of the hydrogens of serum albumin have exchanged with deuterium [pH 5.3 to 6.5, 25° C (101)] and only about 34% to 44% of those in β-lactoglobulin [pH 4.8 to 6.1, 20° C (212)]. Thus, according to hydrogen exchange measurements, serum albumin fluctuates more than either ovalbumin and β-lactoglobulin. Although the examples given are relatively few, they support the idea that proteins able to bind ligands with a high affinity (serum albumin and apomyoglobin) are more readily able to change conformation than those not possessing this property.

The literature cited in this section indicates the existence of various conformational fluctuations of albumin with time constants differing by several orders of magnitude. The large-amplitude motions ("breathing") probably occur at or close to the surface of the protein molecule. These motions probably involve parts of albumin of various sizes, which can move simultaneously or alternately. Ueda and Go (276) proposed a model

according to which various parts of a globular protein can fluctuate independently of one another. This is in accordance with the view of noncooperativity suggested by Benson and Hallaway (12) and Hvidt and Wallevik (101) in the case of serum albumin. The large-amplitude, in contrast to the small-amplitude, motions may play an important role in ligand binding (a more detailed discussion is given in the following section). The small-amplitude motions may occur in both exterior and interior parts of albumin although not necessarily with the same amplitudes because, e.g., various damping mechanisms probably are operative. The detailed nature of the fluctuations of albumin is largely unknown and observations of the motions most often are of a qualitative nature. For example, which parts of the protein are involved in the particular case studied? The range of the conformational changes? The kind of intramolecular binding forces involved in the various fluctuations? Which control mechanisms are operative?

Some of the above-mentioned aspects have been studied in a more detailed and quantitative way in the case of some smaller, globular proteins. X-ray diffraction studies of metmyoglobin have indicated that although all parts of the protein exhibited structural motility, the amplitudes of the fluctuations of the outside residues were, on an average, the double of those carried out by the inside residues (72). Charged and polar residues showed larger displacements than the nonpolar ones. Similar studies on lysozyme have shown that large displacements were associated with segments of the protein not involved in the formation of regular secondary structure and with external side chains (6). This study indicated that long-chain hydrophilic residues and hydrophobic residues were associated with the greatest and smallest motilities, respectively. McCammon et al. (158) calculated that in bovine pancreatic trypsin inhibitor the greatest fluctuations should be assigned to external parts of the protein, whereas deviations from the average conformation were significantly smaller in the interior of the protein. Large displacements were calculated for the atoms in the side chains. However, not only the interatomic distances but also the bond angles change. Nuclear magnetic resonance spectroscopic studies have revealed motility in the interior of pancreatic trypsin inhibitor in the form of rapid rotations of aromatic amino acid residues (295). This phenomenon has also been observed in other globular proteins (295). Furthermore, it was found that the rotation of the aromatic rings is independent of the unfolding equilibria that permit exchange of hydrogen and deuterium atoms (295).

B. Conformational Changes of Albumin Induced by Binding of Ligands

The interaction between albumin and ligands often is accompanied by detectable conformational changes of the protein. Due to the limited information on the three-dimensional structure of albumin the exact nature of the conformational changes, as recorded by various techniques, is difficult to uncover. However, it has been possible to gain insight into various aspects of the ligand-protein interaction by the use of the following techniques.

Optical rotatory dispersion. Binding of several moles of hexyl sulfate (220), deoxycholate (153), or triton X-100 (153) per mole of bovine serum albumin does not change the optical rotation at 233 nm. By contrast, binding of 0 to 10 moles of octyl sulfonate and especially of decyl and dodecyl sulfonate and octyl, decyl, dodecyl, and myristyl sulfate (C_8-C_{14}-sulfates) per mole of bovine serum albumin results in diminished levorotation of the protein, which however was attributed to other conformational changes than a decrease of α-helix content (220). Binding of 0 to 10 moles of dodecanoate per mole of human serum albumin also causes diminished levorotation of that protein, which could be explained by assuming a constant Moffit b_0 parameter and a varying Moffit a_0 parameter (297). This is probably also compatible with a constant content of α-helix of albumin under these conditions.

Fluorescence spectroscopy. Steinhardt et al. (258) have studied the influence of binding of hexyl, octyl, dodecyl sulfate, octyl and dodecyl sulfonate, and dodecanoate on the fluorescence of human and bovine serum albumin excited at 290 nm or 305 nm. At $\bar{\nu} > 2$ to 3 all the ligands change the wavelength of maximal fluorescence of both proteins towards lower wavelengths (blue shifts) to about the same extent. In the case of bovine albumin binding of 1 mole of the ligands results in a *quenching* of the fluorescence, whereas 3 to 4 moles seem to be required before an effect on the fluorescence of human albumin is observed, in the case of which an *enhancement* is registered. The effect on the quantity of the fluorescence is more pronounced with increasing $\bar{\nu}$ (until about 40) and is most evident in the case of the ligands octyl and dodecyl sulfate and dodecyl sulfonate. The authors suggested that the environments of the tryptophan residue(s) in pure albumin solutions are relatively polar. Binding of the alkyl ions changes the environments to apolar ones. The shift in polarity is brought about by conformational changes and/or simple coverage of the residue(s) by one or more of the aliphatic chains of the bound ligand molecules.

Ultraviolet difference spectroscopy. Steinhardt and coworkers have also studied in detail the interaction of alkyl ligands and albumins by ultraviolet difference spectra (211, 219, 259). The ligands especially studied were: C_6-C_{14}-sulfates, C_6-C_{12}-sulfonates, octanoate, decanoate, and dodecanoate. In the following, attention has been focused on the results obtained by nondeionized, undefatted protein preparations. Binding of 1 to 2 moles of the sulfates or sulfonates per mole of human or bovine albumin results in red shifts of the protein spectra (shifts towards higher wavelengths). In the case of native bovine albumin, higher molar ratios result in blue shifts in the

region 250 to 320 nm (aromatic residues) and red shifts below 250 nm (aromatic and/or cysteine residues and/or peptide groups). Binding of the sulfates or sulfonates in these ratios to native human albumin caused red shifts in both absorbance regions. The spectral changes were interpreted in the following way: (1) Binding of 1 to 2 moles of the sulfates or sulfonates to the two proteins perturb tyrosine and phenylalanine (red shifts) rather than tryptophan, which probably is an indication of local changes in the conformation and/or polarity of or in the vicinity of these aromatic residues. (2) At $\bar{\nu} \geq 2$ the difference spectra of bovine albumin furthermore include both tryptophan blue (250 to 320 nm) and red shifts (below 250 nm). These apparently contradictory observations have been explained by assuming conformational changes resulting in transfer of one of the tryptophan residues from a position inside a hydrophobic part of the protein molecule to an exterior, solvent-exposed part and in changes of the vicinity of the other tryptophan residue in a contrary way. For being in accordance with the conclusion based on the fluorescence spectroscopic studies, the effect on the protein fluorescence of the tryptophan residue transferred from a polar to a hydrophobic milieu must be superior to that of the other tryptophan residue. (3) In the case of human albumin ($\bar{\nu} \geq 2$) the spectra also include red shifts in both absorbance regions of the lone tryptophan residue, indicating a more apolar surrounding of the residue. (4) At still higher values of $\bar{\nu}$ (4 to 6 in the case of bovine albumin and ≥20 with the human species) binding of ligands with longer chain lengths (e.g. dodecyl sulfate and dodecyl sulfonate) results in a reversal of the initial red shifts of tyrosine residues to blue shifts. This change in shifts first appears in the region 250 to 320 nm and was suggested to be caused by local conformational changes resulting in transfer of some of the tyrosine residues from protein-buried to solvent-exposed positions. In contrast to binding of the sulfates and sulfonates, binding of the carboxylates results in red shifts of both the proteins in both ultraviolet regions at all molar ratios studied. These spectral changes seem to be caused by perturbation of tyrosine and phenylalanine residues, the milieus of which may become more hydrophobic upon binding. The tryptophan residue(s) do not seem to be involved.

The difference spectra observed, when the ligands mentioned bind to the albumins at various ratios, indicate that a variety of binding sites are involved, which differ among themselves in their perturbing effects on the aromatic amino acid residues (259). The number of high affinity binding sites of the carboxylates is lower than that of the corresponding sulfates and sulfonates (219). It has been proposed that the fatty acids bind to the sites of the other two types of ligands, which are placed in the vicinity of tyrosine residues but not to those in the neighbourhood of both tyrosine and tryptophan residues (219).

Zakrzewski and Goch (297) observed that binding of octanoate, decanoate, and dodecanoate to defatted human serum albumin results in red shifts of the protein difference spectra in the ultraviolet region due to perturbation of tyrosine residues. In accordance with Steinhardt and coworkers, these authors obtained no evidence for the involvement of the tryptophan residue. The first 2 moles of bound fatty acids perturbed the tyrosine residues to a much higher degree than that of the following 5 moles, which in turn caused more pronounced changes in the difference spectra than the very weak bound fatty acid molecules. On the basis of these studies, in combination with optical rotatory dispersion measurements (see above), solvent perturbation investigations and spectrophotometric titration curves of various albumin:fatty acid complexes at pH 10 to 13, it was proposed that binding of fatty acids results in masking of some of the solvent-exposed tyrosine residues and causes local conformational changes resulting in formation of new hydrophobic surfaces on the albumin molecule.

Ryan (225) and Ryan and Gibbs (228) found that binding of testosterone, cortisol, progesterone, and androstenolone results in red shifts of the protein spectra. The red shifts introduced by the four steroids were not identical but were essentially of two different kinds. For example, binding of androstenolone resulted in a shift comparable to that caused by binding of aliphatic fatty acids and was proposed to result from perturbation of tyrosine residues. The other kind of red shifts was represented by binding of testosterone. In this case the spectral changes were different from those introduced by binding of fatty acids and were the result of perturbation of both tyrosine and tryptophan residues. It was suggested that the difference spectra of albumin were the result of local conformational changes of the protein caused by binding of the steroids and/or by formation of hydrogen bonds between ligand and protein.

Electron spin resonance spectroscopy. Conformational changes of bovine serum albumin due to ligand binding have been registered with the aid of nitroxide probes covalently attached to the protein. Blanchard et al. (15) found that binding of several anionic drugs (flufenamic acid, phenylbutazone, sulfaethidole, oxyphenbutazone, and warfarin) resulted in a conversion of the spin-labelled sites from a strongly immobilized to a partially immobilized state. By contrast, binding of cationic drugs (imipramine, desmethylimipramine, and fluphenazine) resulted in a stabilization of the spin-labelled sites, i.e. conversion of partially immobilized to strongly immobilized sites.

Blanchard et al. (15) and Oakes and Cafe (195) have shown that binding of dodecyl sulfate to bovine albumin ($\bar{\nu} < 10$) resulted in an increased mobility of bound spin labels. Addition of dodecyl trimethylammonium chloride at low molar ratios resulted in the same effect (195). Wasylewski and Pasenkiewicz (283) found an increase in

mobility of a bovine albumin-bound spin label accompanying the association of aliphatic sulfates of various chain lengths (C_8-C_{14}). However, at very low ligand concentrations a small decrease in mobility of the spin label was observed.

Hydrogen-deuterium exchange. Benson and Hallaway, with infrared spectroscopy (12), studied the hydrogen-deuterium exchange of bovine serum albumin in the presence and absence of 10 moles of sodium dodecyl sulfate per mole of protein. They observed that binding of the sulfate caused a decrease in the number of fast exchanging hydrogens of albumin at both pH 5.0 and pH 7.7. A corresponding increase in the number of nonexchanging hydrogens was noted while little or no change in rate or number of intermediate exchanging hydrogens was observed. On the other hand, Hvidt and Wallevik (101), with the same technique, found that the exchange rates of human serum albumin at pH 6.1 to 6.3 were not changed by the presence of 10 moles of sodium dodecyl sulfate or 5 moles of potassium palmitate per mole of albumin, respectively. However, at pH 7.62 potassium palmitate reduced the exchange rates of the measurable hydrogen atoms. The effect of dodecyl sulfate was not studied at that pH value.

Hydrogen-deuterium exchange of bovine serum albumin in the absence and presence of various concentrations of Cu^{++} have been studied by Reynolds et al. (218). At pD 4.8 binding of 0 to 4.4 copper ions per albumin molecule did not result in a significant change in the exchange rates of albumin. However, in contrast to binding of dodecyl sulfate (12) and palmitate (101), binding of 10 moles of Cu^{++} resulted in an increase in the number of readily exchangeable hydrogen atoms indicative of a loosening of the native structure of bovine albumin (218).

Volumetry. Katz and coworkers have studied the interaction between bovine serum albumin and dodecyl sulfate (115), thiocyanate (114), trichloroacetate (114), and several metal ions (113, 116) by volumetry. At pH 5.1 binding of dodecyl sulfate to the first high affinity binding sites results in an expansion of the protein, perhaps via a neutralization of positively charged groups of albumin (115). Saturation of the remaining high affinity binding sites causes a pronounced reduction of the volume of albumin. At pH 8.0 binding to all high affinity sites results in a significant reduction in the volume. At high sulfate concentrations ($\bar{v} > 10$) an expansion of albumin is observed at both pH values (115). Binding of trichloroacetate ions is accompanied by an increase of volume at all ligand concentrations studied (114). At the same pH (5.0), binding of thiocyanate at low concentrations causes a small reduction in volume, whereas at high concentrations an increased volume of albumin is observed (114). The interaction of albumin and 13 different metal ions is associated with, quantitatively varying, volume increases (113). A *decreased* volume of albumin due to binding of ligands with an aliphatic moiety probably is the result of hydrophobic associations between the ligand and the protein. Such associations may result in a "tightening" of parts of the albumin molecule involving a conversion of fluctuating parts to more rigid structures. Sturtevant (260) suggested that such a conversion takes place when glyceraldehyde-3-phosphate dehydrogenase interacts with NAD^+. The volume *increases* accompanying binding of small ligands, especially the metal ions, probably can be explained by changes in hydration and/or protonation at the binding site and/or changes of protein conformation. Recently, Katz et al. (116) published a detailed study correlating data obtained by volumetry and equilibrium dialysis of binding of Cu^{++} to bovine albumin. Changing pH from 5.3 to 7.4 resulted in an increased Cu^{++} binding but in a relatively smaller increase in the volume of the protein. The experimental data at pH 5.3 can be explained without assuming conformational changes of the protein. By contrast, at pH 7.4 binding of only one Cu^{++} per albumin molecule seems to be able to change the conformation of the protein. Binding of the two first Cu^{++} probably results in unmasking of charged groups. Binding of more ligand molecules induces a structural transition, probably more widespread, as evidenced by a cooperative binding isotherm.

Conformational stability of albumin by ligand binding. Several observations indicate a stabilization of the albumin molecule when ligands are reversibly bound to it. For example, fatty acids protect the protein against the action of heat (19, 24, 86), while defatted albumin is unstable on storage (269). Dodecyl sulfate, at molar ratios of ligand to protein below 10, reduces the expansion of albumin at high pH values (150) and protects it against denaturation by heat (86) and urea (155). Reynolds et al. (220) also suggested a stabilization of bovine serum albumin when less than 10 moles of dodecyl sulfate are bound to the protein. Furthermore, testosterone, androsterones, and some other steroids, Ca^{++}, and small amounts of ethanol are able to inhibit the hydrolysis of human and bovine serum albumin by trypsin (226). Markus (154) and Markus et al. (156) observed that binding of L-tryptophan (154), methyl orange, crystal violet, and some other dyes (154, 156) to human albumin diminishes the rate of digestion of the protein by various enzymes. Binding of pentachlorophenol, 2,4-dinitrophenol, 2,4,6-trinitrophenol, dicoumarol, and other ligands results in a decrease of the proteolysis of bovine albumin by trypsin and pronase (286). It is important to note that in all cases the stabilizing effect is observed when only 1 to 2 ligand molecules are bound per albumin molecule. Furthermore, it should be noted that the effect is achieved upon binding of ligands probably interacting with different binding regions and that the protein is protected against denaturating agencies of different nature.

Markus et al. (156) studied in more detail the digestion of human serum albumin by trypsin and chymotrypsin

in the presence and absence of various ligands. Binding of negatively charged azo dyes not only reduced the rate of enzymatic digestion but also resulted in formation of mixtures of albumin fragments varying with the ligand in question and different from digestion of pure albumin. King (119) observed that addition of octanoate results in the formation of mainly large fragments when bovine serum albumin is digested by pepsin. Proteolysis in the absence of octanoate resulted in a complex mixture of small fragments. Despite the aforementioned examples, ligand binding is not always accompanied by a lesser tendency for proteolytic attack. For example, association of positively charged triphenylmethane dyes caused an *increment* in the tryptic digestion rate and resulted in formation of other combinations of protein fragments (156). Ryan (226) found that binding of progesterone and several of its derivatives, cortisol and cholestane, also *increased* the rate of enzymatic digestion of bovine serum albumin. Binding of progesterone and cortisol *increased* the proteolysis of human serum albumin (226).

Cooperative and antagonistic binding of ligands to albumin are other manifestations of ligand-introduced conformational changes. Cooperativity can be homotropic (i.e. binding of identical ligands) or heterotropic [i.e. binding of different ligands (171)]. Some examples of heterotropic cooperativity can be given. For example, binding of progesterone and testosterone to albumin is enhanced by the presence of 3 moles of palmitate per mole albumin (227). Binding of progesterone is also increased in the presence of 5 moles of laurate (227). Bilirubin binding to human albumin was increased when 1 to 3 moles of laurate, myristate—and possibly also—palmitate were added per mole of albumin (30). The cooperative effect on binding of bilirubin to bovine albumin was much more pronounced and was also observed in the presence of decanoate and oleate (30). Furthermore, addition of long-chain fatty acids in molar ratios to human albumin of about 3 to 4 increases the binding of warfarin (43, 44, 237, 291). Recently, Katz et al. (116) reported the rather surprising finding that binding of Cu^{++} to bovine albumin at pH 7.4, in contrast to binding at pH 5.3, is cooperative as studied by equilibrium dialysis. Antagonistic binding of two different ligands has been proposed to be the case when, e.g., low concentrations of long-chain fatty acids decrease the binding of chlorophenoxyisobutyrate (254), halofenate (254), and diazepam (241). A more detailed description of the conformational changes accompanying cooperative and antagonistic binding is not possible on the basis of the above-cited literature and the information needed for such a description probably cannot be furnished by simple binding experiments.

It is largely unknown how great a part of the albumin molecule is involved in a conformational change caused by binding of a ligand molecule. However, Santos and Spector (230, 231) have shown that binding of a ligand placed in domain 3 (palmitate) can alter the fluorescence of a ligand probably bound in domain 2 (1-anilino-8-naphthalene sulfonate). The domains are numbered according to a 3-domain albumin model (fig. 4). According to equilibrium dialysis measurements, the conformational modification of or at the 1-anilino-8-naphthalene sulfonate binding site was not so pronounced that the binding percent was changed (or the ligand was bound to the modified binding site with the same association constant). Furthermore, Reed (214) proposed that the increased binding of bilirubin (domain 2) to defatted bovine albumin caused by addition of long-chain fatty acids (domain 3) is brought about by conformational changes of the protein.

The results mentioned above may be explained as follows. Binding of anionic ligands results in a more compact structure and/or in a restriction in the degrees of freedom of groups of the albumin molecule especially in the vicinity of the binding sites. For example, binding of more than a few moles of dodecyl sulfate to bovine albumin (but with $\bar{\upsilon} < 10$) results in a reduction of the volume of the protein (115). Furthermore, binding of aliphatic ligands may result in an increase in the number of nonexchangeable hydrogen atoms (12, 101). This "tightening" of the albumin structure probably is the reason for the lesser tendency for proteolytic attack. Binding of cationic ligands apparently results in a "looser" structure of albumin. An increase in the overall volume of albumin is registered upon interactions between the protein and various metal ions (113, 116), and binding of 10 copper ions results in a detectable increase of the number of readily exchangeable hydrogen atoms (218). The increased enzymatic digestion rate accompanying binding of, e.g., positively charged triphenylmethane dyes (156) could be the result of a "looser" albumin structure caused by binding of the dyes.

According to electron spin resonance spectroscopic studies, the "tightening" introduced by binding of negatively charged ligands seems to be accompanied by a concurrent "loosening" of the structure in other parts of the protein. By contrast, binding of positively charged drugs results in a reduced mobility of spin-labelled parts of the albumin molecule. The changes in mobility detected by electron spin resonance spectroscopy may be due to some of the ligand-introduced conformational changes responsible for not only the changed enzymatic digestion rates but also for the changed fragmentation pattern.

The following two theories of the influence of ligand binding on the conformation of albumin can be proposed: (1) Albumin in solution is not a system of identical molecules but a collection of isomeric proteins of approximately equal energy and in thermodynamic equilibrium with each other (112, 149). Addition of a ligand to the protein solution results in preferential interaction between the ligand and the isomeric form of albumin re-

sulting in the most stable complex (ligand-isomer combinations with the highest association constants). (2) The flexibility of albumin is so pronounced that a ligand molecule, during the binding process, is able to modify the configuration of a binding region in such a way that a greater congruence between the ligand molecule and parts of the region (the binding site) is achieved ("adaptability" of albumin).

Chen (47) studied the binding of bilirubin to bovine and other albumins with the stopped-fw technique. Measurements of the protein fluorescence quenching and the appearance of bilirubin fluorescence suggested the existence of the following 3 steps: (1) A very rapid, initial contact between the two reactants takes place resulting in a fast decrease of protein fluorescence. (2) Bilirubin changes conformation from a nonfluorescent, random conformation to a fluorescent, chiral form. (3) Then a secondary reduction in protein fluorescence occurs probably indicating conformational changes of albumin or perhaps a rotation of the bound bilirubin. An alternative, very fascinating, explanation for the secondary quenching is a migration of bilirubin along a binding crevice (47). The secondary quenching of protein fluorescence was not observed upon binding to human albumin. A possible reason for this difference is that bilirubin could be bound closer to one or both of the tryptophan residues in bovine albumin than to the single residue in human albumin. Later, Færch and Jacobsen (73) studied the kinetics of bilirubin binding to human albumin by stopped-flow measurements of the absorbance of bilirubin. These authors subdivided the interaction according to the following model: simple association followed by four consecutive steps. The results of Reed (214) and Gray and Stroupe (83) also are in accordance with the observations that binding of bilirubin to albumin includes the existence of intermediate steps.

Recently, Scheider (235) studied the binding of oleate to human albumin also by a stopped-flow technique. He proposed that the binding involves two steps. The first step is diffusion-controlled and probably consists of an attachment of the fatty acid in a nonspecific manner. The second step is rate-limited by a negative entropy of activation and involves a rearrangement of the protein-fatty acid complex. In this step the ligand is probably transferred to a hydrophobic interior of albumin.

Adams and Berman (1) carried out a detailed kinetic investigation of the interaction of haemin with human serum albumin in dimethyl sulphoxide/water (3:5, v/v). These authors proposed that the haemin-albumin complex is formed by a two-stage process. The first step should be a chemically controlled process and involves an interaction of haemin with a group on the protein surface with a pK-value of 5.9 (a histidine residue?). The formation of the intermediate ligand-protein complex is followed by an entropy-controlled internalization of the haemin molecule.

The results of the above-cited kinetic studies seem to be in accordance with the idea of albumin adaptability.

The adaptability of the binding regions of albumin, however, is not unlimited. Certain steric requirements have to be fulfilled by the ligand before binding with a high association constant can occur. This is apparent from binding studies with groups of substituted organic ligands such as benzoates (265), phenols (265), indoles (164), phenothiazines (74, 97), and benzodiazepines (238, 243). In all of these studies it was observed that some, relatively simple, substituents in certain positions increased the binding affinity, whereas the same substituents placed in other positions decreased binding. That the adaptability of albumin is not unlimited is also evident from the considerations represented in section IV, "Specificity of Albumin Binding."

C. *Binding of Ligands to the Same Region*

The proposal of the existence of six or more binding regions of albumin is based on observations, where $K_A' = K_B' = 0$ (equations 3 and 4 in "Appendix"). This situation can be brought about by competitive binding to identical sites and perhaps also to overlapping sites. However, it is possible that such a blockage can be caused by other mechanisms. Binding of ligand *A* could result in conformational changes of albumin sufficient to disrupt or mask the binding site of *B*.

Examples of inhibition through conformational changes can be given. In myoglobin the haem group is placed in a hydrophobic pocket of the globin part of the molecule. Lind and Møller (147) removed the haem group and studied the simultaneous binding of several pairs of ligands, bound with a high affinity, to the "empty" binding region (apomyoglobin). In the presence of haem or protoporphyrin IX the binding of bromophenol blue was inhibited according to a competitive scheme ($K_A' = K_B' = 0$). However, the inhibition was proposed to be caused by conformational changes of the binding region introduced by binding of haem or protoporphyrin IX and not by competition for the same site.

When two ligands bind simultaneously to albumin it is not compelling to conclude that they are bound to different regions. Examples of simultaneous binding of 2 ligands to parts of albumin occupied by 1 molecule of another ligand can be given. Soltys and Hsia (249) suggested that 2 molecules of the spin label GABA-DNB-SL (see fig. 6A) can bind to the region accommodating 1 molecule of bilirubin. Lind and Møller (147) observed that in apomyoglobin 1 molecule of bromophenol blue and bilirubin or dodecanoate can be bound in the pocket associating only 1 molecule of haem or protoporphyrin IX. However, unless the binding regions are very large as compared with the size of the ligands, changes in the association constants of the two ligands appear probable.

In conclusion, the results obtained on the binding of two ligands according to a competitive scheme and the

various examples of independent binding presented in the literature, however, provide strong evidence for the existence of more binding regions on the albumin molecule. This hypothesis is further supported by the studies of ligand binding to modified albumins and to fragments of the protein.

APPENDIX

MATHEMATICAL ANALYSIS OF SIMULTANEOUS BINDING OF TWO LIGANDS

When two ligands are bound by a protein, the association of each ligand can be influenced by the presence of the other ligand. In the following we shall first assume that there is only one binding site for each ligand, A and B, by the protein P. If binding conforms to a competitive scheme the following equations are obeyed:

$$\bar{\nu}_A = \frac{K_A A_f}{1 + K_A A_f + K_B B_f} \quad (1)$$

$$\bar{\nu}_B = \frac{K_B B_f}{1 + K_B B_f + K_A A_f} \quad (2)$$

where $\bar{\nu}_A$ and $\bar{\nu}_B$ are the average number of molecules of A and B, respectively, bound per molecule of protein; K_A and K_B are the association constants of A and B, respectively; A_f and B_f are the concentrations of free ligand.

If A and B are simultaneously bound, this may lead to a change in association constants by one or more of the following mechanisms: (1) Conformational changes of the protein induced by binding of each ligand; (2) electrostatic interactions between charged ligands (long-range forces as compared with hydrophobic attractive forces); (3) binding of the two ligands close to each other at the same binding region (overlapping binding sites resulting in partially competitive binding). In this case, the following equilibria may exist:

$$P + A_f \overset{K_A}{\rightleftharpoons} PA$$

$$PB + A_f \overset{K_A'}{\rightleftharpoons} PBA$$

$$P + B_f \overset{K_B}{\rightleftharpoons} PB$$

$$PA + B_f \overset{K_B'}{\rightleftharpoons} PAB$$

Under these conditions $\bar{\nu}_A$ and $\bar{\nu}_B$ are given by:

$$\bar{\nu}_A = \frac{K_A A_f \;\; 1 + K_B B_f}{K_A A_f + 1 + K_B' B_f} \quad (3)$$

$$\bar{\nu}_B = \frac{K_B B_f \;\; 1 + K_A A_f}{K_B B_f + 1 + K_A' A_f} \quad (4)$$

Competitive binding of A and B is characterized by $K_A' = K_B' = 0$. By inserting these values in equations 3 and 4, the relationships are reduced to those of equations 1 and 2. Independent binding of A and B is observed when $K_A' = K_A$ and $K_B' = K_B$. In this case equations 3 and 4 are altered to:

$$\bar{\nu}_A = \frac{K_A A_f}{1 + K_A A_f} \quad (5)$$

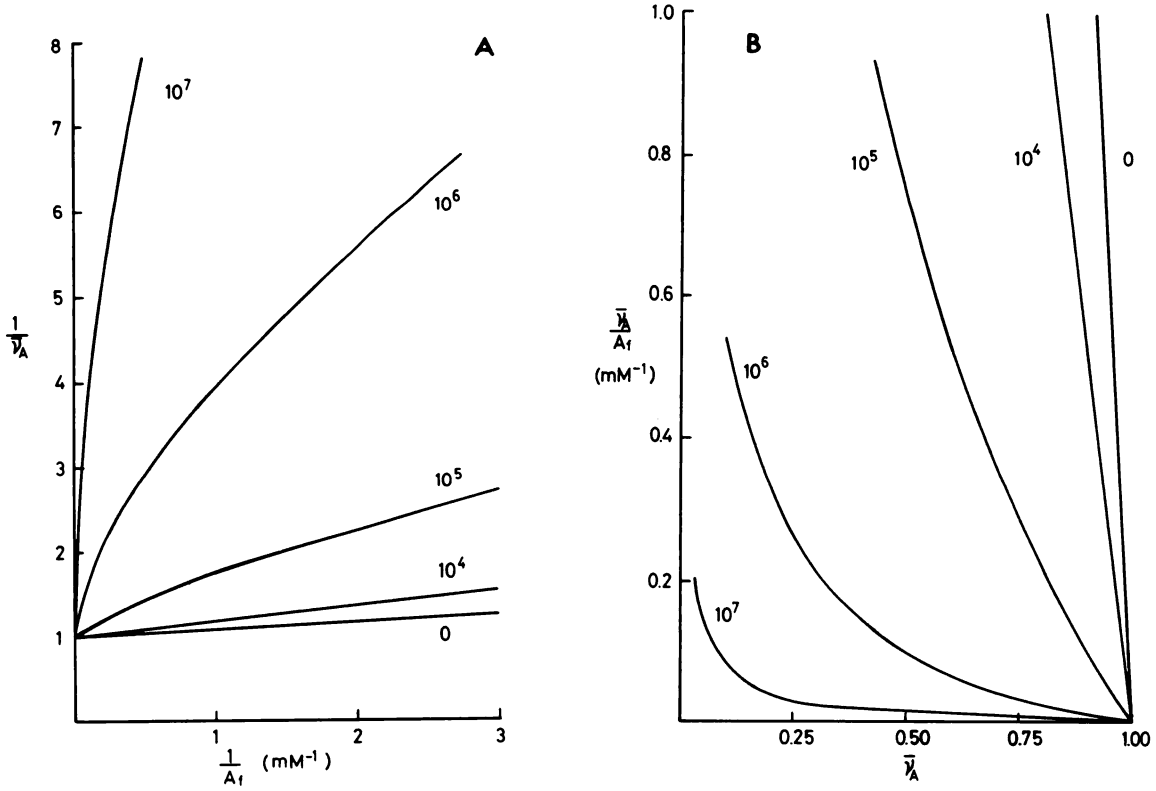


FIG. 9. Theoretical examples of competitive binding of two ligands (A and B) to one site of serum albumin. The *total* concentrations of B and of albumin are constant (10^{-4} M), whereas the *total* concentration of A varies. The association constant of A is the same in all the examples given (10^4 M^{-1}), whereas that of B varies as indicated in the figures. 0 denotes binding of A in the absence of B. $\bar{\nu}_A$ and A_f represents the average moles of A bound per mole of albumin and the concentration of *free* A, respectively. Figure 9A, data plotted according to equation 7; figure 9B, the same data plotted according to equation 8.

$$\bar{\nu}_B = \frac{K_B B_f}{1 + K_B B_f} \quad (6)$$

In all other cases, binding of A and B cannot be explained simply as competitive or independent, but is influenced by direct or indirect effects as mentioned above. Association of B can result in an increase ($K_A' > K_A$) or a decrease ($K_A' < K_A$) of binding of A, and vice versa. We note that if $K_A' = K_B' = 0$ this is indistinguishable from competitive binding, in which case $\bar{\nu}_A$ and $\bar{\nu}_B$ reach their minimal values.

In practice, the possibility of competitive binding of two ligands to one binding site can be examined in different ways. First of all the concentrations of free A and B have to be determined. Methods such as equilibrium dialysis, ultrafiltration, and gel chromatography can be used depending on the nature of the ligands in question.

A widely used method for treating the experimental data is the double-reciprocal plot, equation 7, which is an analogue to the Lineweaver-Burk plot. However, that plot analyzes the displacement of a protein-bound ligand in the presence of a great surplus of the inhibitor. In this case, the error introduced setting the total concentration of inhibitor B equal to the free concentration is small. Making this approximation when the total concentrations of A, B, and P are comparable, which is usually the case when studying simultaneous binding of two ligands to albumin, invalidates the analysis.

The following rearrangement of equation 1 can be made:

$$\frac{1}{\bar{\nu}_A} = \frac{1}{A_f}\left(\frac{1 + K_B B_f}{K_A}\right) + 1 \quad (7)$$

An analogous modification of equation 2 can be carried out. An examination of equation 7 reveals that a linear dependency of $1/\bar{\nu}_A$ and $1/A_f$ cannot exist at any values of K_A and K_B, because the figures in the parenthesis are not constants (B_f varies as a function of A_f). Figure 9A shows hypothetical examples of $1/\bar{\nu}_A$ plotted against $1/A_f$. K_A and the total concentrations of B and P are constant, whereas K_B and the total concentration of A are varied. It is seen that a linear dependency is approached only in the case when A and B are bound to the common site on the protein with similar binding constants. In this case, however, the concentration of free inhibitor B approaches that of total inhibitor, and the binding curve of A is not much different from that calculated without inhibitor.

A similar conclusion is drawn when the data are plotted according to the method of Scatchard (233), see figure 9B and equation 8.

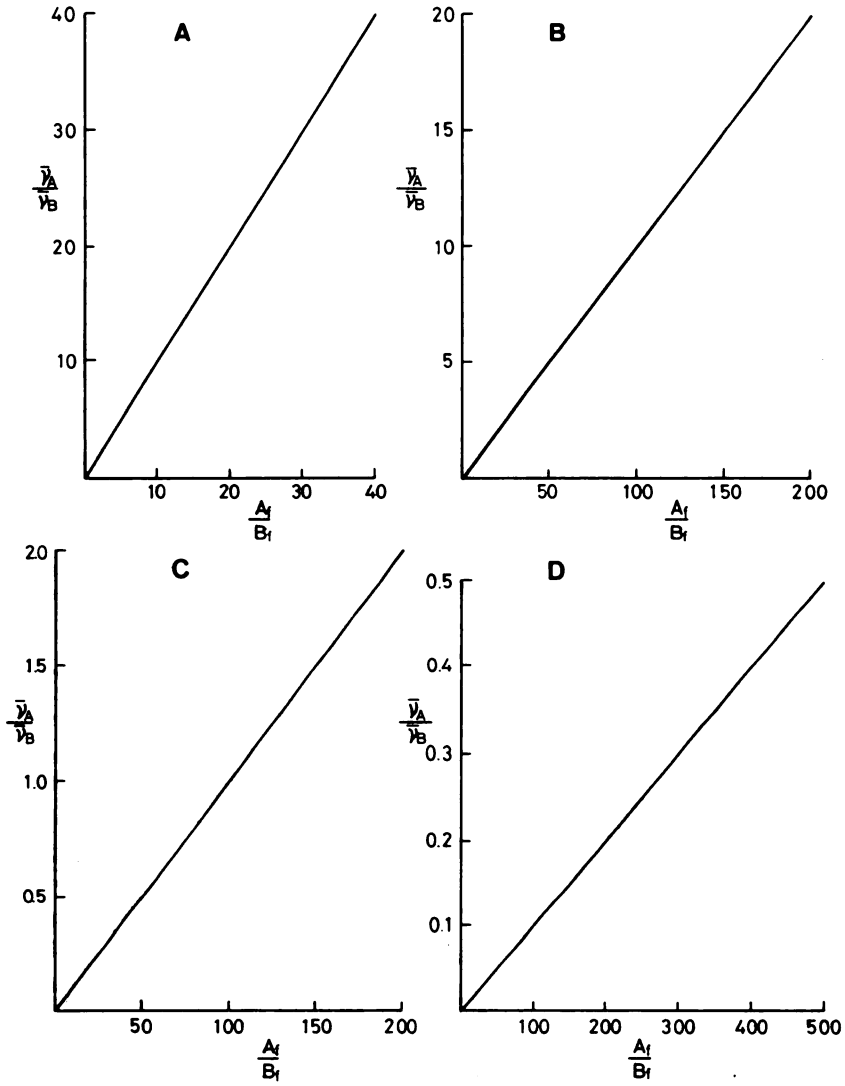


FIG. 10. Theoretical examples of competitive binding of two ligands (A and B) to one site of serum albumin. The same data as in figures 9A and 9B but plotted according to equation 10. Figure 10A, association constant (K_B) of ligand $B = 10^7\ M^{-1}$; figure 10B, $K_B = 10^6\ M^{-1}$; figure 10C; $K_B = 10^5\ M^{-1}$; figure 10D; $K_B = 10^4\ M^{-1}$. $\bar{\nu}_A$ and $\bar{\nu}_B$ represents the average moles of A and B, respectively, bound per mole of albumin. A_f and B_f denote the concentration of *free* A and B, respectively.

$$\frac{\bar{\nu}_A}{A_f} = 1 - \bar{\nu}_A\left(\frac{K_A}{1 + K_B B_f}\right) \quad (8)$$

Equation 1 can also be rearranged to a form analogous to a Dixon-Webb plot:

$$\frac{1}{\bar{\nu}_A} = B_f\left(\frac{K_B}{K_A A_f}\right) + \frac{1 + K_A A_f}{K_A A_f} \quad (9)$$

Equation 2 can be rewritten in a similar way. As in the case of equation (7) the figures in the parenthesis are not constants. Thus, proportionality of $1/\bar{\nu}_A$ and B_f cannot exist.

As an alternative to reciprocal plots, another way of linear analysis of the data has been proposed (129). The ratio of equations 1 and 2 results in the following relationship:

$$\frac{\bar{\nu}_A}{\bar{\nu}_B} = \frac{K_A}{K_B} \times \frac{A_f}{B_f} \quad (10)$$

It is seen that $\bar{\nu}_A/\bar{\nu}_B$ is proportional to A_f/B_f for any pair of ligands bound to one site of a protein. In figures 10A to 10D theoretical examples are given based on the same conditions as used in constructing figures 9A and 9B. Regression lines are observed for all the examples given and the slopes of the lines are equal to the ratios of K_A and K_B. These values can be checked by determining the ratio of the binding constants of A and B, respectively, without the presence of the other ligand.

In principle, the possibility of competitive binding to one site can be examined when the total concentration and not the free concentration of one of the ligands, say B, is known. The relation between the concentration of total B (B_t) and free B is:

$$B_t = B_f + \bar{\nu}_B P_0 \quad (11)$$

where P_0 is the concentration of the protein. Let us assume that binding is competitive and that B is not bound elsewhere on the protein. In that case $\bar{\nu}_B$ can be calculated according to the following equation obtained by combining equations 1 and 2:

$$\bar{\nu}_B = 1 - \bar{\nu}_A\left(\frac{1 + K_A A_f}{K_A A_f}\right) \quad (12)$$

Insertion of $\bar{\nu}_B$ into equation 11 gives B_f, and the binding can be analyzed according to equation 10. It should be realized that this method of analysis presupposes that B does not interact with other binding sites on the protein. Hence it is very desirable in these experiments to measure simultaneous binding of the two ligands.

The analysis of ligand binding becomes more complicated when there are several binding sites for ligands A and B with different association constants as is generally the case for the albumin molecule. In this case the study of simultaneous binding to one high affinity binding site requires ligand-protein ratios below unity for minimizing binding to secondary sites. At higher ligand-protein ratios (>1) some of the binding sites may be common while others may be different. The overall result may probably be a partial inhibition of binding of the other ligand, an effect which in practice can be indistinguishable from that obtained by indirect effects mediated by binding at different sites. In this case, a first approach can be to estimate the total number of binding sites of A and B, $n_{(A+B)}$, at high ligand concentrations, and to compare this number with that obtainable by binding of each ligand alone, $n_A + n_B$. However, this procedure cannot identify the specific binding site for A that is in common for the particular binding site for B. A theoretical approach to this problem has been presented by Edsall and Wyman (62). Dealing with analysis of binding data in terms of competitive binding to more binding sites can be eased by the use of computer programs.

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