review

Fatty acid binding to plasma albumin

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Abstract A review of the available information about fatty acid binding to plasma albumin is presented. Albumin is composed of a single polypeptide chain, folded so as to form three or four spherical units. The strong fatty acid binding sites probably are located in crevices between these spherical regions. The anionic form of the fatty acid binds to albumin. Most of the binding energy comes from nonpolar interactions between the fatty acid hydrocarbon chain and uncharged amino acid side chains that line the binding sites. The binding sites are somewhat pliable, and their configuration can adapt to fit the incoming fatty acid. Stepwise association constants for binding to human albumin of fatty acids containing 6–18 carbon atoms are presented. These data indicate that each mole of fatty acid binds with a different affinity and that the association constants for multiple binding diminish sequentially, i.e., $K_1 > K_2 > K_3 > \ldots > K_n$. Because of uncertainties concerning fatty acid association in aqueous solutions, the constants for the 14–18 carbon acids probably are not definitive. In the usual physiological concentration range, free fatty acids do not displace appreciable amounts of a second organic compound from albumin. Sensitive spectrophotometric analyses revealed, however, that even small increases in free fatty acid concentration alter the molecular interaction between human albumin and another organic compound.

Supplementary key words drug binding · plasma transport · fluorescence · tryptophan · free fatty acid · proteins · anionic detergents · organic dyes · lipoproteins

When Kendall crystallized human serum albumin in 1941 (1), he observed that the product contained a small amount of free fatty acid (FFA). Others noted that the lipids extracted from blood plasma also contained small quantities of FFA. The physiological significance of these observations was not recognized until 1956. In the intervening period, however, studies of FFA binding to albumin were carried out for several reasons. First, plasma albumin came into use clinically, and fatty acids were noted to stabilize the protein against denaturation (2). Therefore, it was of practical importance to learn how FFA exerted this protective effect. Second, Scatchard had just described his classical work on protein binding (3), and it was only natural that these concepts be extended to organic anions, including fatty acids.

The first indication that FFA binding to albumin might be important metabolically came from studies of the lipemia-clearing reaction (4, 5). Subsequently, Korn showed that albumin was the acceptor for the FFA released by the heparin-activated lipoprotein lipase (6, 7). Additional work by Dole (8), Gordon and Cherkes (9), Gordon (10), and Laurell (11) demonstrated that plasma FFA had an extremely fast turnover rate and were rapidly responsive to metabolic and nutritional changes. Finally, Gordon and Cherkes (12) and White and Engel (13) showed that albumin was the transport vehicle for FFA released from adipocytes. These findings established the central role of FFA binding to albumin in the mammalian lipid transport process.

ALBUMIN STRUCTURE

Only those structural aspects that are essential for an understanding of the fatty acid binding process will be considered.

Chemical properties

Albumin consists of a single polypeptide chain of molecular weight 67,000 ± 2000 (14). Bovine plasma albumin is estimated to have between 566 and 621 amino acid residues (15–17), the most accurate values currently available being 579[1] and 587 (15). Human albumin contains between 569 and 613 amino acid residues (16, 18). Albumin is a spherical molecule that has a large net negative charge at physiological pH.

Abbreviations: FFA, free fatty acid(s); K_i, the association constant for binding to the ith site; ANS, 1-anilino-8-naphthalenesulfonate; c, the unbound fatty acid concentration in molarity units; $\bar{\nu}$, the molar ratio of bound fatty acid to albumin; n_i, the number of individual sites in the ith class of binding sites; k_i^j, the average apparent association constant for the individual sites that constitute the ith class of binding sites.

[1] Peters, T., Jr. Personal communication.

Even the most pure preparations of crystalline plasma albumin are not homogeneous (19). One cause of the heterogeneity is oxidation of the single free sulfhydryl group. Most albumin preparations contain only 0.6 mole of reduced sulfhydryl groups per mole of protein (20). This is especially important for fatty acid binding studies because one of the main fatty acid binding sites is located near the single reduced sulfhydryl group. Oxidation of this cysteinyl sulfhydryl group alters the binding of fatty acids at this site (20). Pairing of disulfide bonds also accounts for heterogeneity (21). In addition, there may be differences in the primary structure of albumin monomers (19). Therefore, it is likely that some protein impurities are present even when the most pure crystalline albumin preparations are used.

Plasma albumins contain only one (human) or two (bovine) tryptophan residues. This greatly enhances the usefulness of spectroscopic methods for studying the molecular mechanism of binding. Many other aromatic residues are present, e.g., 30 phenylalanines and 18 tyrosines in human albumin. Yet, tryptophan alone is almost entirely responsible for the ultraviolet fluorescence of the protein (22). The albumin fluorescence spectrum is perturbed when long-chain fatty acids or anionic detergents are bound (23, 24). This affords the possibility of mapping fatty acid binding sites relative to the locations of the tryptophan residues. Conformational changes that accompany binding also can be followed by measuring tryptophan fluorescence. Additional information can be obtained by investigating the effects of fatty acids on the emission spectrum of a fluorescent probe. This is done by exciting the tryptophan residues of albumin and measuring the influence of added fatty acids on energy transfer to the fluorescent probe (25). Human albumin is particularly amenable to study by these techniques because it contains only one tryptophan residue, making interpretations somewhat less complicated.

Conformation

According to the structural model for bovine plasma albumin proposed by Pederson and Foster (15), the polypeptide chain contains four globular regions. Each of these regions is stabilized covalently by disulfide linkages. According to Pederson and Foster (15), albumin behaves as if it were composed of subunits even though it actually consists of only a single polypeptide chain. Another model, proposed by Anderson and Weber (26), consists of three spherical regions. The four binding sites with highest affinity for organic anions are located in hydrophobic crevices perpendicular to the juncture of the central and peripheral spheres. This model is identical with the one proposed by Bloomfield (27) in which the central sphere has a radius of 26.6 Å and the two peripheral spheres have radii of 19.0 Å. The currently available binding data are compatible with either the three- or four-sphere structural models.

Amino acid sequence

Recent primary sequence data suggest a repeating structure for the albumin molecule (28). The sequence Cys-Cys occurs seven times, and the spacing of other Cys residues on either side of these seven sequences is similar. Two disulfide bonds are located in a carboxyl-terminal fragment that contains 76 amino acid residues (29). From this information, the location of the other 15 disulfide bonds was determined. Brown (28) has interpreted these findings as indicating that the albumin molecule is composed of nine domains, many of which have similar amino acid sequences and which, therefore, probably arose through gene duplication. This repeating structure is consistent with the presence of groups of equivalent or nearly equivalent fatty acid binding sites, a concept proposed originally by Goodman (30). From his analysis of fatty acid binding to human albumin, Goodman concluded that there are three different classes of albumin binding sites (30). The sites within a given class have nearly equal affinities for fatty acids, suggesting that they probably are structurally similar. Our studies of fatty acid binding to albumin do not support the existence of groups of nearly equivalent sites because the association constant (K_i) for the binding of each successive mole of fatty acid is different (31–34). Our findings can be reconciled with the Goodman model, however, by postulating that one mole of fatty acid influences the binding of the next through cooperative effects. This would make the K_i values for two moles of fatty acid different even if they bind to structurally similar sites.

These questions concerning the nature of the fatty acid binding sites should be resolved shortly because crystals suitable for X-ray diffraction studies have become available (35). The most useful crystals for diffraction work are those of the monoclinic form prepared from human albumin. They contain four molecules per unit cell, and their highest diffraction resolution is 2.7 Å.

Species differences

Because of its easy availability and low cost, bovine albumin has served as the reference protein for most binding studies. It is important to know the extent to which results obtained using bovine albumin apply to other albumins, particularly to human albumin. Comparative studies of palmitate binding to bovine, human, and rabbit albumins indicated that only small differences occurred in the binding constants (bovine > rabbit > human) (36). More detailed studies using fluorescence spectroscopy, however, revealed basic qualitative differences between various albumins in terms of molecular changes associated with fatty acid binding (23, 37, 38). Studies with 1-anilino-8-naphthalenesulfonate (ANS) indicated that rabbit and bovine albumins exhibited similar fluorescence changes. These changes differed from those noted with canine and human albumin (37). Such species differences with respect to fatty acid binding actually are not surprising. For ex-

ample, although homologous regions exist in the amino-terminal regions of human and bovine albumin, 6 of the first 24 amino acids differ in these proteins (39). Human albumin undergoes a much greater conformational change than bovine albumin when the pH is raised from 6.9 to 9.3 (40). Conversely, larger optical rotatory changes accompany dye binding to bovine albumin (41). Energy transfer from tyrosine to tryptophan residues is greater in human than bovine albumin (42). Finally, spectroscopic studies with 2-(4'-hydroxyphenylazo)-benzoate suggest that there are structural differences among these as well as other albumins (43, 44). Taken together, these results indicate that there are important differences in the molecular interactions between fatty acids and plasma albumins from various species. Therefore, one must be cautious in extrapolating albumin binding data from one species to another.

MECHANISM OF BINDING

Most of the information available on the mechanism of binding has been obtained with organic dyes, anionic detergents, and fluorinated or spin-labeled derivatives, not fatty acids. There are differences in the mechanism of binding fatty acids as compared with other organic anions, at least for the high affinity binding sites of human albumin (31, 45). Yet, the information obtained with the model compounds about many aspects of the binding process almost certainly applies to fatty acids as well. Therefore, I will summarize briefly some of the main conclusions from the studies with model compounds.

Hydrophobic interactions

The strength of binding of the alkyl sulfate detergents increases as the length of the alkyl chain increases (46). Dye binding also increases as the number of hydrocarbon groups in a ring structure increases (47, 48). Moreover, an uncharged derivative of methyl orange, aminoazobenzene, binds almost as well as methyl orange itself (49). On the other hand, when hydrocarbons contain anionic groups, an interaction occurs between the anions and certain positively charged residues of albumin, such as the ϵ-amino groups of lysine (48). Most of the binding energy in such cases is contributed by the nonpolar interactions, not the electrostatic interactions (50, 51).

These findings have led to the generally held view that hydrophobic interactions account for most of the binding energy when large organic anions such as fatty acids bind to albumin. Only a small enthalpy change accompanies organic anion binding to albumin. By contrast, there is a positive entropy change of 14 to 17 e.u. for binding of the first mole of an organic anion (52). Therefore, binding is primarily an entropy effect that probably results from release of water when the anion–protein complex forms. Studies with palmitate and stearate analogs containing spin-labeled nitroxyl groups also indicate that the binding energy is derived primarily from hydrophobic interactions (53). The hydrophobic interactions between the hydrocarbon groups and the albumin binding site extend along the entire length of the fatty acid chain. Methyl esters of the spin-labeled analogs were bound somewhat less tightly than the corresponding carboxylate anion (53), indicating that electrostatic or hydrogen bonding interactions involving the carboxylate group also occur in fatty acid binding. The hydrophobic interactions are much more important, however, and the carboxylate group probably plays only a minor role when fatty acids bind to the strongest albumin sites.

Fluorine magnetic resonance studies with trifluoroalkyl sulfates have provided additional insight into the molecular mechanism of the binding process (54). They indicate that the ω-terminus of the hydrocarbon chain is not held firmly in place in the binding site. This suggests that the strongest hydrophobic interactions probably occur along the acyl chain, not at the deepest point of penetration of the chain into the hydrophobic binding pocket. Moreover, these studies indicate that the electrostatic interaction does not fix the hydrocarbon tail rigidly in place within the hydrophobic binding site. When the trifluoroalkyl sulfates were bound to albumin at low molar ratios, no ligand–ligand interactions were observed. This suggests that at physiological concentrations only one mole of an organic anion is contained within each albumin binding site and that all of the hydrophobic interactions occur between the hydrocarbon chain and nonpolar amino acid side chains that line the binding pocket.

Heterogeneity of the binding sites

When Scatchard plots (3) are applied to either dye or anionic detergent binding data, the relationship is nonlinear (46, 55). Electrostatic interactions are much too small to account for the nonlinearity. Therefore, the albumin binding sites must be heterogeneous (55, 56). Most of the dye and detergent binding data can be resolved in terms of two classes of albumin binding sites. The very large positive entropy changes noted above occur only for binding to the strong class of sites.

Configurational adaptability

When more than 10 moles of dodecyl sulfate is added to albumin, a major change in the protein conformation occurs (57). Many new binding sites become available, and new electrophoretic species appear. Gross conformational changes such as this do not occur under physiological conditions.

A much more subtle conformational change, known as configurational adaptability, accompanies binding in the physiological range (58). This concept was developed by Karush (55, 59). He showed that the albumin binding sites are pliable and can take on different configurations having nearly equal energies. A given organic anion stabilizes that configuration which permits maximum interac-

tions between itself and the binding site. The binding sites have configurational adaptability because the intrahelical attractions in albumin are weak, permitting the amino acid side chains to assume different orientations. According to this concept, the large positive entropy change results from rupture of intramolecular bonds as the configuration of the site changes. As binding occurs, the helices separate, and new binding sites are created. This is a major difference between the Karush and the Scatchard binding models. The Scatchard model assumes that albumin contains a fixed number of preexisting sites that compete independently for available ligand (3). By contrast, the Karush model assumes that binding sites are created and modified as binding occurs, accounting for conformational changes and cooperative binding effects. The Karush model is much more consistent with the currently available experimental data for fatty acid binding to albumin.

RATE OF BINDING

Ott (60) measured the rate of long-chain FFA binding to albumin using a stop-flow spectrophotometric method. The measurements had to be made at pH 11.5 because the method was based on fatty acid-induced changes in the ionization of the tyrosine hydroxyl groups. Fatty acid binding was found to be a second-order reaction with a $t_{1/2}$ of 11 msec. Although the physiological rate of binding also must be very rapid, I have some reservations about using the values obtained by Ott because the measurements were made at such a high pH. Plasma albumins undergo several structural changes over the pH range. An isomerization known as the N–F transition occurs between pH 3.5 and 4.5 (61). A change in tertiary structure occurs between pH 7.5 and 9.0 (62). Expansion of the albumin molecule occurs between pH 10 and 11, and irreversible denaturation occurs in the pH range of 11.4–13.0 (63). Organic anion binding is independent of pH between 4.8 and 6.8 (64), but the binding of at least some anions, including octanoate (32, 65), is altered when the pH exceeds 7.5 (48, 59, 64). Therefore, Ott's measurements should be considered as estimates rather than as precise values for the physiological rate of FFA binding to albumin.

SPECIFICITY OF BINDING

Few proteins besides albumin can bind FFA, and none can bind them as tightly or in such large amounts. Of the commercially available proteins that have been tested (66, 67), only β-lactoglobulin is able to bind appreciable quantities of fatty acid. On a weight basis, β-lactoglobulin can bind only half as much fatty acid as albumin, and its affinity for fatty acids is only about 1% that of albumin. Plasma low density lipoproteins also can bind FFA (68), but they begin to compete effectively with albumin for fatty acids only when the molar ratio of FFA to albumin exceeds 4, such as after an injection of heparin (69, 70). However, lipoproteins have a relatively high affinity for fatty acids that contain 20–24 carbon atoms, and they compete effectively with albumin for these very long chain acids (71). Several intracellular proteins also have some capacity to bind fatty acids, especially the cytoplasmic fatty acid-binding protein, which probably is synonymous with Z protein (72, 73), fructose 1,6-diphosphate-1-phosphatase (74), and acetyl coenzyme A carboxylase (75, 76).

The FFA extracted from human plasma albumin contains at least 43 separate fatty acids. The physiologically important 16 and 18 carbon atom fatty acids account for 90% of the FFA present in these extracts (77). Likewise, oleate, palmitate, linoleate and stearate make up most of the FFA present in crystalline bovine albumin preparations, with oleate present in the largest amounts (20). Commercial albumin preparations usually contain small amounts of short-chain acids as well, especially citrate, pyruvate, and lactate (78).

FATTY ACID BINDING SITES

The organic anion binding sites of albumin are composed of two parts, a pocket lined with nonpolar amino acid side chains and a cationic group located at or near the surface of the pocket (79). FFA binding involves hydrophobic interactions with the hydrocarbon chain and electrostatic interactions with the carboxylate anion. The presence of nonpolar interactions were indicated by the observations that fatty acids stabilize the albumin tertiary structure against denaturation by heat, urea, or guanidine hydrochloride (80, 81). Evidence for electrostatic interactions was obtained from electrophoretic measurements (82) and binding studies in which the cationic residues of albumin were modified with acetic anhydride, *O*-methyl isourea, or formaldehyde (36). When albumin is heavily modified by acetylation or amidation, however, its tertiary structure is altered (83). Therefore, some of the reduced binding thought to result from removal of cationic groups actually may result instead from conformational changes. Moreover, agarose affinity columns containing palmityl aminoalkyl chains bind albumin tightly (84). This is due entirely to nonpolar interactions, for the fatty acid carboxyl group is covalently linked to the aminoalkyl agarose support. Finally, uncharged fatty acid analogs such as methyl palmitate and hexadecanol bind to albumin, but to a lesser degree than palmitate (85, 86). Therefore, although the electrostatic interactions probably contribute somewhat to the binding energy, they are not essential for binding.

As predicted by the studies with dyes and detergents, there is no gross structural disorganization when fatty acids in physiological amounts bind to albumin (87).

However, subtle structural changes do occur. FFA binding produces a volume increase together with a decrease in the axial ratio and dipole moment of the albumin molecule (88). This is due primarily to nonpolar interactions between the fatty acid tail and the binding site and is a reflection of the configurational adaptability of the binding sites (55, 59). When the fatty acid hydrocarbon chain penetrates into the interior of the globular albumin molecule, the helices separate as the binding sites adapt to incoming hydrocarbon chain structure. This produces a small change in the tertiary structure of the protein. These conformational changes result in greater susceptibility of some tyrosine residues to iodination (89) and diminished reactivity of several amino groups to dinitrophenylation (90).

Swaney and Klotz (79) have shown that one of the strong organic anion binding sites of human albumin contains the amino acid sequence: Lys-Ala-Trp-Ala-Val-Ala-Arg. The five nonpolar side chains form the hydrophobic pocket, and a cationic group is present at each of the ends. This type of structure is supported by dinitrophenylation studies showing that two lysine residues are located near fatty acid binding sites of bovine albumin (90). Arginine residues also have been shown to be present near the strong organic anion binding sites of bovine albumin (91). The fatty acid binding sites are probably located in clefts between the globular regions of the albumin polypeptide chain (26, 92).

Localization of the tryptophan residues

When organic anions bind to albumin, the fluorescence of the tryptophan residues is perturbed. The simplest interpretation is that tryptophan forms a part of the nonpolar binding site, as in the structure described by Swaney and Klotz (79). It is equally possible, however, that the tryptophan perturbations are due to fatty acid-induced structural changes resulting from the configurational adaptability mechanism.

Human albumin contains only one tryptophan residue. Its fluorescence is quenched by iodide ions (20) and ANS (38). Testosterone, cortisol, and clofibrate appear to bind to the tryptophan-containing site (45, 93), i.e., to the structure isolated by Swaney and Klotz (79). It is likely, however, that the first 2 or 3 moles of fatty acids do not combine with this site. These quantities of fatty acid perturb the tyrosine absorbance spectrum of human albumin, but not that of tryptophan (94, 95). They also have essentially no effect on tryptophan fluorescence (23). Low concentrations of palmitate do not displace ANS from this site even though the first mole of palmitate is bound to albumin over 100 times more tightly than ANS (38). Palmitate reduces the quenching produced by iodide, but the reduction is progressive until 4 moles of palmitate are bound (45). Fluorine magnetic resonance studies suggest that only 1 mole of fatty acid is present at each binding site and that the sites are separated, i.e., there are no ligand–ligand interactions unless very large amounts of FFA are bound (54). Therefore, palmitate cannot protect the lone tryptophan residue by a steric effect. The protection probably is indirect, being due to a progressive conformational change induced by palmitate binding to other regions of the albumin molecule.

If more than 2 or 3 moles of fatty acid is added to human albumin, the tryptophan fluorescence is enhanced (95) and ANS is displaced from albumin (38, 45). When the primary binding sites of albumin are occupied, the excess fatty acid appears to spill over and only then begins to occupy the site containing the lone tryptophan residue. Therefore, the structure isolated by Swaney and Klotz (79) probably is *not* one of the two or three primary binding sites for FFA.

The situation is more complex in bovine albumin because it contains two tryptophan residues. When fatty acid binds, there is quenching and a blue shift of the tryptophan fluorescence spectrum (23). Both changes are progressive until 4–5 moles of fatty acid is added. Two different processes probably are responsible for these fluorescence changes (24). Quenching results from alteration in the state of ionization of a protein ϵ-amino group, whereas the blue shift is due to movement of one tryptophan residue into a less polar environment. Spectrophotometric studies indicate that the alkyl chain of the bound fatty acid is located near tyrosine residues, not the tryptophan residues (96). These observations suggest the following explanation for the fluorescence effects. One of the tryptophans of bovine albumin is located deep inside the globular structure, whereas the other is superficially located and fairly accessible to solvent (20). Several of the strong fatty acid binding sites are located within 10 Å of the buried tryptophan residue, but the alkyl chains bound at these sites do not interact directly with the tryptophan residue. The configurational adaptability that accompanies FFA binding to these sites alters the environment of this buried tryptophan residue, leading to progressive quenching as the strong binding sites are filled. The blue shift in the fluorescence spectrum results from movement of the second, or superficial, tryptophan residue into an environment that is more protected from the solvent. This change also is progressive and, hence, secondary to structural changes associated with FFA binding at other locations in the albumin molecule.

FATTY ACID ASSOCIATION IN AQUEOUS SOLUTION

Fatty acid anions in monomeric form bind to plasma albumin (30, 82). In order to calculate the binding constants, one must measure the amount of the anionic form that is bound as well as the concentration of unbound monomeric anions in the solution.[2] The unbound fatty acid is

[2] In theory, c represents an activity and not a concentration. The difference between the two values is very small, and concentrations are almost always used instead of activities in binding studies.

composed of anions and undissociated acid. In practice, the total unbound fatty acid concentration is measured and is considered to be the unbound anion concentration (c). This approximation is considered to be valid when binding is measured at pH 7 or higher because the pK_a of fatty acids is thought to be about 4.8. Some measurements of the pK_a of long-chain fatty acids, however, gave much higher values. The available data fall into two categories: measurements made in pure aqueous solution that give pK_a values of 6–8 for the long-chain fatty acids (97–99), and mixed solvent titrations that give pK_a values in the expected range of 4.7–5.0 (100–102). I suspect that the mixed solvent titrations probably give the more correct values. On theoretical grounds, lengthening of the hydrocarbon chain should have very little inductive effect on the carboxyl group, so it is difficult to explain why long-chain fatty acids should have much higher pK_a values than short- or medium-chain acids. In addition, association of undissociated acid probably occurs at low pH in pure aqueous solutions, especially when the fatty acid concentrations are high. If these aggregates do not ionize easily, the subsequent titration would appear to have a high pK_a value. If the high pK_a values observed for long-chain fatty acids are artifacts as I suspect, then the actual pK_a values probably are between 4.8 and 5.0 (100–102). Therefore, at pH 7.4, the ratio of anions to undissociated acid is about 780, and the total unbound fatty acid concentration is a valid approximation for c.

Goodman's (103) studies of fatty acid partition, using a pK_a value of 4.8, revealed unexplained anomalies with fatty acids containing 14 or more carbon atoms. Mukerjee (104) postulated that anion dimerization in the aqueous solutions produced these anomalies. According to his analysis, dimerization occurs much below the critical micellar concentration, i.e., at about 1 μM with stearate. Smith and Tanford (105) also observed FFA association in the low concentration range, but they think that the aggregates contain fatty acid anions and the undissociated acid. According to physical chemical theory, the free energy of fatty acid transfer from water to heptane should increase linearly as the fatty acid chain length increases. Deviations from linearity occurred in the analyses of both Goodman and Mukerjee when the alkyl chain exceeded 13 carbon atoms (103, 104), and this could not be explained. In Smith and Tanford's analysis, however, the free energy of transfer remains linear even when the alkyl chain contains 21 carbon atoms, the longest acid that they tested (105). Our studies concerning the existence of premicellar association are inconclusive (106), but they raise doubts about the validity of both Mukerjee's (104) and Smith and Tanford's (105) explanations. A major reason for the present uncertainty as to whether fatty acid association occurs below the critical micellar concentration is that the pK_a values for the long-chain acids are still open to some question (97–102), and pK_a is a term in the partition equation (103–106). If, as some believe (97–99), the pK_a values of the long-chain fatty acids are much higher than 4.8, all of the partition data can be made to fit the standard partition equation without any need for the presence of an aqueous phase association term (106).

If appreciable fatty acid association occurs in the 1–50 μM concentration range, as suggested by the work of Mukerjee (104) and Smith and Tanford (105), all of the currently available albumin binding data for the 16 and 18 carbon atom acids are incorrect because the total unbound FFA concentration would not be an accurate approximation of the unbound FFA monomer anion concentration. If the magnitude of aqueous association reported by Smith and Tanford (105) is correct, the reported K_i values for palmitate and stearate would be about five times too small.

ASSOCIATION CONSTANTS

Short- and medium-chain fatty acids

Teresi and Luck (107) measured the binding to bovine albumin of fatty acids containing 2–8 carbon atoms by equilibrium dialysis. These data were analyzed according to the Scatchard model (3):

$$\bar{\nu} = \frac{n_1 k_1 c}{1 + k_1 c} + \frac{n_2 k_2 c}{1 + k_2 c} + \cdots + \frac{n_n k_n c}{1 + k_n c} \quad \text{Eq. 1}$$

where $\bar{\nu}$ is the molar ratio of bound fatty acid to albumin, n_i is the number of binding sites in the ith class, k_i is the average apparent association constant for the sites in the ith class, and c is the concentration of unbound fatty acid anions (3). The best-fitting Scatchard model contained two classes of binding sites, $n_1 = 4.2$–5.0 and $n_2 = 27$–31. The k_i values increased as the chain length of the acid increased; i.e., k_1 at 23°C was 80 M^{-1} for acetate, 550 M^{-1} for hexanoate, and 6500 M^{-1} for octanoate (107).

The binding of hexanoate, octanoate, and decanoate to human albumin defatted with activated charcoal (87) also has been measured by equilibrium dialysis (32). These data were analyzed by the stepwise equilibrium model

$$\bar{\nu} = \frac{K_1 c + 2K_1 K_2 c^2 + \cdots + NK_1 K_2 + \cdots + K_N c^N}{1 + K_1 c + K_1 K_2 c^2 + \cdots + K_1 K_2 + \cdots + K_N c^N} \quad \text{Eq. 2}$$

where K_i is the individual association constant for each mole of the ligand. As opposed to the Scatchard analysis, there is no grouping of the individual binding sites into classes in this formulation. The K_i values for binding of the first 5 moles of the medium-chain FFA are listed in **Table 1.** Decreasing K_i values were obtained with multiple binding, i.e., $K_1 > K_2 > \ldots > K_n$. The K_i increased as the fatty acid chain length increased, i.e., hexanoate < octanoate < decanoate.

When either palmitate or oleate was added, octanoate binding was reduced (32). This suggests that medium- and

TABLE 1. Association constants for fatty acid binding to human plasma albumin

Binding Site	K_i								
	Hexanoate	Octanoate	Decanoate	Laurate	Myristate	Palmitate	Stearate	Oleate	Linoleate
					M^{-1}				
1	1.5×10^4	3.4×10^4	1.0×10^5	2.4×10^6	2.1×10^7	6.2×10^7	1.5×10^8	2.6×10^8	7.9×10^7
2	2.4×10^3	9.7×10^3	2.8×10^4	1.1×10^6	6.4×10^6	2.3×10^7	5.3×10^7	9.4×10^7	8.7×10^6
3	3.1×10^2	4.5×10^3	2.0×10^4	4.9×10^5	2.7×10^6	1.2×10^7	1.9×10^7	2.9×10^7	5.1×10^6
4	2.0×10^2	1.4×10^3	1.2×10^4	2.5×10^5	1.0×10^6	3.1×10^6	5.6×10^6	2.1×10^7	3.1×10^6
5	4.7×10	1.6×10^3	7.8×10^3	1.9×10^5	8.1×10^5	1.5×10^6	4.5×10^6	1.1×10^7	6.8×10^5

Incubations were done at 37°C in a buffer containing 122 mM NaCl, 4.9 mM KCl, 1.2 mM $MgSO_4$, and 16 mM Na_2HPO_4 adjusted to pH 7.4 with 0.1 N HCl. These data are presented in more extensive form in Refs. 32 and 34. The values are calculated assuming that *no* fatty acid association takes place in the aqueous solution and that the pK_a of the fatty acids is 4.8.

long-chain FFA compete for the same albumin binding sites. Binding of octanoate was not altered when the pH was reduced from 7.4 to 6.5, but it decreased when the pH was raised to 8.2 (32). These pH-induced changes are similar to those observed with other organic anions (59, 64) and probably result from structural changes in the albumin molecule (63).

Long-chain fatty acids

For unexplained reasons, fatty acids containing 12 or more carbon atoms will not pass through ordinary dialysis membranes at pH 7.4. Because of this, their binding to proteins in the physiological pH range cannot be studied by equilibrium dialysis. All binding measurements with long-chain FFA are made by the equilibrium partition method, in which the protein solution is incubated with fatty acid dissolved in heptane. Goodman made the first measurements with the 12–18 carbon atom fatty acids (30). His studies were done at 23°C and pH 7.4 using human albumin preparations that were defatted with a mixture of isooctane and glacial acetic acid (108). Based upon a Scatchard analysis (3), Goodman (30) divided the albumin binding sites into three classes; his data for palmitate are given in **Table 2.** Also listed in Table 2 are the palmitate binding data obtained by Arvidsson, Green, and Laurell, who made measurements at pH 8.5 (109). Considering the difference in pH, the Scatchard model obtained by Arvidsson et al. (109) is remarkably similar to Goodman's (30) results.

We also have studied the binding of the 12–18 carbon atom fatty acids (33, 34, 36). Our measurements were made at pH 7.4 using albumin preparations that were defatted with activated charcoal (87). Scatchard analyses of our palmitate binding data for human and bovine albumin are presented in Table 2. Although certain of the details differ, the results are basically similar to those reported by Goodman. Recently, we made more sophisticated measurements with human albumin using newer data for fatty acid partition between heptane and aqueous solutions (106). K_i values obtained by a stepwise equilibrium analysis for binding of the first 5 moles of the long-chain FFA are given in Table 1. These values were calculated assuming that no FFA association occurred in the aqueous solution (106). In agreement with Goodman's findings (30), we also observed that oleate was the most tightly bound of the long-chain FFA and that binding of the saturated acids increases as the chain length increases from 6 to 18 carbon atoms. If binding were simply a statistical process limited only by the number of available hydrocarbon groups in the fatty acid alkyl chain, it should increase linearly with chain length. Plots of the log K_i against fatty acid chain length are S shaped, not linear (34). The largest deviations from linearity occur between decanoate and laurate. This could be due to a methodological artifact, for the medium-chain FFA were studied by equilibrium dialysis (32) whereas laurate and the longer acids were studied using the more complex equilibrium partition method involving heptane (34). Hydrocarbons are known to bind to albumin (110) and, perhaps, influence the ability of albumin to combine with another ligand. Direct comparison of the dialysis and partition methods, however, indicated

TABLE 2. Scatchard models for palmitate binding to plasma albumin

Albumin	Temperature	pH	Binding Parameters						Reference
			n_1	k_1	n_2	k_2	n_3	k_3	
	°C			M^{-1}		M^{-1}		M^{-1}	
Human	23	7.4	2	6.0×10^7	5	3.0×10^6	20	1.0×10^3	30
Human	25	8.5	2	8.0×10^7	5	4.0×10^6	30	2.0×10^4	109
Human	23	7.4	2	5.2×10^7	4	9.2×10^6	20	9.1×10^2	36
Bovine	23	7.4	3	2.9×10^7	3	6.0×10^5	63	6.6×10^2	36
Bovine	37	7.4	3	6.8×10^6	3	5.0×10^5	63	9.3×10^2	36
Human	37	7.4	3	4.9×10^6	3	2.8×10^5	63	3.5×10^2	36

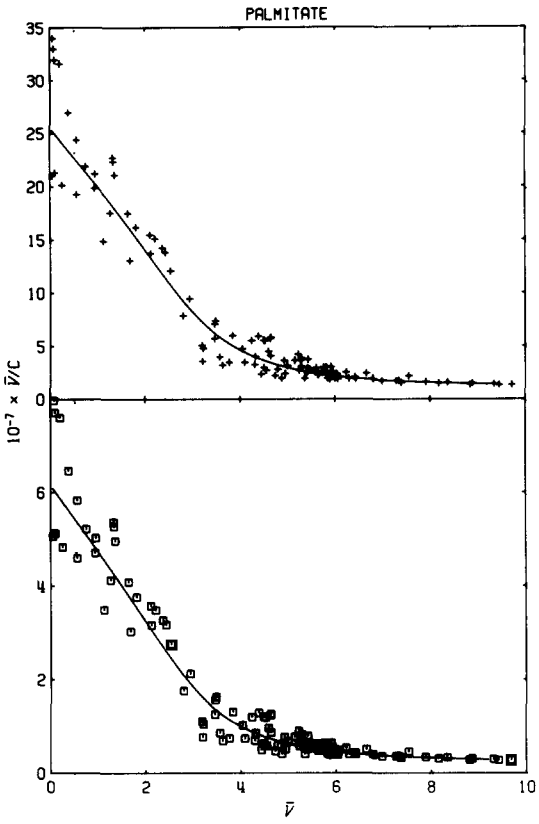

Fig. 1. Palmitate binding to human plasma albumin. The upper plot shows the data that were calculated assuming the existence of palmitate association in the aqueous solution; the lower plot shows the same data calculated without taking palmitate association into account. The binding measurements were made at 37°C by the equilibrium partition method (36) under the conditions listed in Table 1. Calculations for palmitate association were made using the values given in Ref. 106.

that they give similar results with decanoate (34). The equivalence of the two methods also has been reported for testosterone binding (111). Recalculation of the myristate, palmitate, and stearate data to account for aqueous association also did not correct the deviation from linearity in these log K_i plots (34). I believe that the nonlinear log K_i relationship is real and reflects differences in the configurational adaptability of the albumin binding sites. According to this view, adaptability is greatest for laurate, leading to a high percentage of the potential binding interactions actually being expressed. Although palmitate and stearate have more hydrocarbon groups for potential binding interactions, the limited plasticity of the albumin binding sites prevents as large a percentage of the additional binding contacts from actually taking place. Therefore, the increment in binding energy relative to laurate is less than would be predicted from the increase in the number of hydrocarbon groups.

Our data for long-chain fatty acid binding to charcoal-defatted bovine albumin also were fitted to stepwise equilibrium binding models similar to the one shown for human albumin in Table 1 (33). Palmitate was bound more tightly by bovine than human albumin, but laurate and oleate were bound more tightly by human than bovine albumin (36).

Effect of fatty acid association in aqueous solution

Fig. 1 illustrates the data and the best-fitting isotherm for palmitate binding to human albumin. The upper plot shows the results when fatty acid association is taken into account; the lower plot shows the same data calculated under the assumption that no association occurs. There are large differences in these two data sets. For example, the intercept on the y-axis is over 4 times greater when association is taken into account (upper plot). The values for association in these calculations were obtained from our partition measurements (106). If the partition data of either Mukerjee (104) or Smith and Tanford (105) were used, an even greater discrepancy between the two data sets would result. The K_i values for myristate, palmitate, and stearate binding to human albumin when fatty acid association is accounted for are listed in **Table 3.** When these values are compared with the corresponding ones in Table 1 (no FFA association), very large increases are noted, particularly in the K_i for palmitate and stearate. In a previous study with bovine albumin, we concluded that fatty acid association would have very little effect on the binding constants if the FFA concentration was in the physiological range (33). Our more recent partition results (106) indicate that this conclusion was incorrect. It is now clear that major binding differences will result even at low FFA concentrations if fatty acid association is a real phenomenon. The key question of whether or not it is a real phenomenon is still unsettled. Until this is resolved conclusively, all data concerning the *quantitative* aspects of long-chain FFA transport, uptake, and utilization must be regarded as tentative.

Cooperative binding

Since the K_i values for a given fatty acid occur in decreasing order (Tables 1 and 3), it is unlikely that large cooperative effects are associated with multiple binding of FFA to albumin. It generally is accepted, however, that the binding of 1 mole of ligand will influence the binding of subsequent moles in a protein containing multiple binding sites. Karush's concept of configurational adaptability (55, 59) actually implies cooperativity, and the data of Arvidsson et al. (109) suggest that site–site and ligand–ligand interactions do occur when long-chain fatty acids bind to albumin. Physical measurements showing that small changes in shape accompany FFA binding (88) are also compatible with some degree of cooperativity in the binding process. The existence of negative cooperativity could reconcile our findings of everywhere decreasing K_i values

(Tables 1 and 3) with Goodman's concept of groups of nearly equivalent binding sites (30), a view strengthened by the recent demonstration of repeating amino acid sequences in the albumin primary structure (28). For example, if the first two binding sites actually are similar or identical, forming an n_1 class (30, 60, 94), the fact that K_1 is considerably larger than K_2 may reflect an inhibitory effect of fatty acid at the n_1 site on the binding ability of the n_2 site. Likewise, there is evidence suggesting that positive cooperativity occurs at some of the sites even though it is too small to completely reverse the downward trend in the K_i, e.g., at K_3 for decanoate, palmitate, and linoleate and at K_4 for hexanoate, decanoate, myristate, oleate, and linoleate (Table 1). Taken together, these considerations suggest that FFA binding to albumin is a very complex process in molecular terms and that any simple explanation such as the Scatchard model (3) is a gross oversimplification of the actual binding mechanism.

TABLE 3. Association constants for fatty acid binding to human plasma albumin, calculated by assuming the existence of fatty acid association in the aqueous solution

Binding Site	K_i		
	Myristate	Palmitate	Stearate
		M^{-1}	
1	2.5×10^7	2.6×10^8	9.1×10^8
2	7.6×10^6	9.8×10^7	3.6×10^8
3	3.2×10^6	5.4×10^7	1.3×10^8
4	1.3×10^6	1.4×10^7	4.3×10^7
5	1.0×10^6	7.4×10^6	3.5×10^7

Experimental conditions are the same as those listed in Table 1. Corrections for fatty acid association were made using the data in Ref. 106.

Selection of a binding model

Four models have been proposed for the analysis of FFA binding to albumin. The Scatchard model (Eq. 1) groups the albumin binding sites into distinct classes (3). Each class contains a number of individual sites that have nearly equal affinities for fatty acids. The stepwise or multiple equilibrium model (Eq. 2), proposed for protein binding by Klotz, Walker, and Pivan (47), was applied to fatty acids by Fletcher, Spector, and Ashbrook (31). Arvidsson et al. (109) have derived a model in which all of the albumin binding sites are assumed to have the same strength on the average. Fatty acids are not distributed randomly among the sites, however, because of site–site and ligand–ligand interactions. Laiken and Némethy (112) have proposed a model that bears some resemblance to statistical mechanical theories of polymer adsorption at a surface. The fatty acid can bind in a number of different configurations according to this formulation, and not all of the alkyl chain is in contact with the binding site in many of these configurations. Variable contacts occur because the alkyl chain is flexible, giving rise to conformational entropy, which alters the binding equilibrium.

Although Arvidsson et al. (109) and Laiken and Némethy (112) have obtained excellent fits of fatty acid binding data using their respective models, these models have not as yet received widespread acceptance. Most investigators still employ the Scatchard analysis, primarily because it can be carried out using a simple graphical procedure (3). This method is perfectly adequate when the protein contains only one high-energy binding site for the small molecule. Several problems arise, however, when it is used to analyze fatty acid binding to albumin. Eq. 1 was derived by assuming that each of the multiple binding sites of the macromolecule preexists and competes independently for the available ligand. Therefore, the theoretical basis of the model is violated by physical phenomena that accompany fatty acid binding, such as configurational adaptability (55, 59), cooperativity (88), and site–site or ligand–ligand interactions (109). Practical problems also can arise when the binding of two or more fatty acids is compared using Eq. 1. For each class of sites, k_i depends on the value selected for n_i. In order to compare the binding of two or more acids, one must fit their data to the same Scatchard model, e.g., $n_i = 2, 5, 20$ as proposed by Goodman (30). Such a generalized model usually is a compromise and not the best-fitting model for each fatty acid (30, 36). In addition, when $n_i > 1$, k_i is an average constant, not a true association constant for each mole of bound ligand (113). Use of the average k_i values to calculate thermodynamic binding parameters has led to serious errors (36). Finally, the commonly used graphical extrapolation of the Scatchard binding isotherm to obtain the total number of binding sites of the macromolecule is experimentally and mathematically invalid (46, 113).

All of these objections are overcome by the stepwise equilibrium model (113). Eq. 2 is completely general and applies to all multiple equilibrium reactions independently of mechanism. No assumptions are made as to the preexistence or independence of binding sites. Therefore, the model can account for configurational adaptability, positive or negative cooperativity, site–site interactions, or ligand–ligand interactions. True association constants, K_i, are obtained for each mole of bound ligand, and one can compare the binding of two or more different compounds on a mole-for-mole basis without having to assume any uniformity in the binding process. Because of this, the best-fitting binding isotherm for each compound is always used, rather than a compromise fit. The only drawback of the stepwise equilibrium model is that a sophisticated computer is needed for the analysis, which requires a nonlinear least squares fitting procedure (113). By contrast, the Scatchard analysis can be performed graphically in a few minutes. In spite of this convenience, the Scatchard model can no longer be considered acceptable in cases

where multiple binding occurs, such as with FFA and albumin. These studies should be analyzed using either Eq. 2 or one of the more complex, newer models (109, 112).

ALBUMIN FRAGMENTS AND SYNTHETIC POLYMERS

Another approach to understanding FFA binding to plasma albumin is to perform binding studies with albumin fragments rather than with the intact protein. King and Spencer (114) and King (115) have examined the binding of octanoate to fragments of bovine albumin, and Peters (116) and Feldhoff and Peters (117) have investigated the binding of palmitate to similar fragments. The results with octanoate suggest that the primary nonpolar binding site of albumin is located in the carboxyl-terminal fragment containing amino acid residues 330–579. Two possible explanations are advanced as to why k_1 is smaller for octanoate binding to this fragment than to intact albumin. One is that the conformation of the fragment may change when it is removed from the intact protein. The other is that portions of the native three-dimensional binding site may be contributed by residues contained in the remainder of the molecule. The latter possibility is supported by the observation that k_1 for octanoate increases when the amino-terminal fragment is added to the isolated carboxyl-terminal fragment (115). A complex of these two fragments actually forms when they are mixed together (115). A somewhat similar picture is obtained from studies with palmitate.[1] Most of these strong binding sites are also contained in the carboxyl-terminal fragment. At least one moderately strong palmitate binding site, however, is present in the amino-terminal fragment containing residues 1–329. With palmitate, however, k_1 for binding to the carboxyl-terminal fragments is not enhanced when the isolated amino-terminal fragment is added.

As noted with octanoate (114, 115), none of the isolated fragments binds palmitate quite as firmly as the intact albumin molecule. This suggests that some of the amino acid residues that contribute to fatty acid binding are located in different parts of the primary structure. They are brought close together only in the native conformation. This view is consistent with the model proposed by Anderson and Weber (26), in which the high-affinity binding sites are located in crevices formed by the contact of adjacent globular regions of the polypeptide chain.

Studies with synthetic polymers have also provided additional insight into the binding mechanism. Polylysine, which has an extended, open conformation in aqueous solution, binds methyl orange very poorly (118). When polylysine is thiolated with thiobutyrolactone and the sulfhydryl groups are oxidized, it becomes a very compact structure due to extensive cross-linking by disulfide bridges. In this form, it binds methyl orange to a much greater extent. Albumin, like cross-linked polylysine, also has a very compact conformation. As in polylysine, the compact conformation allows nonpolar side chains of albumin to cluster and thereby form hydrophobic binding pockets (118). Polymers that can bind methyl orange even more tightly than albumin have been synthesized (119). These are polyethylenimine derivatives with butyryl, hexanoyl, lauroyl, carbobenzoxytyrosine, or carbobenzoxytryptophan side chains. Like cross-linked polylysine and albumin, they also have very compact conformations in aqueous solution. Strong cooperative interactions occur as these polymers take up increasing amounts of methyl orange. In addition to serving as model compounds for studies of hydrophobic binding, these synthetic polymers ultimately may be useful as lipid transport substances.

COMPETITIVE BINDING BETWEEN FATTY ACIDS AND OTHER ORGANIC COMPOUNDS

Albumin serves as the plasma transport protein for many other organic compounds besides FFA. These include physiological metabolites, such as bilirubin (120) and bile acids (121); hormones, such as thyroxine (122) and testosterone (93, 111), which are transported primarily by globulins and bind to albumin only when they are present in very high concentrations; and drugs, such as salicylates (123), warfarin and dicoumarol (124, 125), digitalis (126), sulfonilamides (127), antibiotics (128), and clofibrate (45). Even under ordinary conditions, the molar ratio of FFA to albumin in human plasma varies between 0.5 and 1.5 (129). In special situations, such as after vigorous muscular exercise (130, 131) or an injection of heparin (70, 132), the molar ratio can exceed 4. Therefore, it is important to determine whether the ability of albumin to transport a second compound can be influenced by changes in the plasma FFA concentration.

Experimental model compounds

Cogin and Davis (133) examined the effects of oleate, stearate, and elaidate concentrations on the binding of methyl orange to bovine albumin. 3 moles of methyl orange was added initially to the albumin. Addition of up to 2 moles of FFA caused almost no displacement of the dye, suggesting that albumin has separate binding sites for the first 2 moles of fatty acid. When more than 2 moles of FFA was added, methyl orange was displaced in greater than mole-for-mole amounts. Cogin and Davis (133) suggested that binding of the third or fourth moles of FFA produced steric hindrance at the dye binding sites leading to the displacement of more than mole-for-mole quantities of the dye. In my opinion, a more likely explanation for the displacement of more than mole-for-mole amounts of methyl orange is a fatty acid-induced conformational change in the dye binding sites.

Goodman (30) also concluded that the main FFA binding sites were separate from the dye binding sites of human albumin. He observed that methyl orange was not

displaced from albumin when up to 2 moles of palmitate was added. Based upon this, Goodman concluded that the two strongest FFA binding sites, which he grouped into a single class, were specific for long-chain FFA and excluded other organic ligands. He also concluded that the secondary class of four or five albumin binding sites was available to FFA as well as other organic compounds (30).

Our findings with human albumin, using the fluorescent probe ANS as the model compound, are in general but not total agreement with the ideas put forward by Goodman. We also noted that 1–2 moles of palmitate or oleate did not influence ANS binding as measured by equilibrium dialysis (38). However, even these small amounts of long-chain FFA altered the ANS fluorescence spectrum. The fluorescence changes are presumed to reflect FFA-induced structural changes in the ANS binding sites (secondary sites). The extent to which binding at the secondary sites is altered appears to depend in part on the structure of the ligand being bound (45). In agreement with the results of Cogin and Davis (133), we also observed that the addition of 3 or more moles of FFA displaced large quantities of ANS from albumin (37, 38).

Drugs

Rudman, Bixler, and Del Rio (134) measured the effects of palmitate and oleate on the binding of salicylate, bromsulfophthalein, phenylbutazone, sulfadiazine, thiopental, bishydroxycoumarin, and diphenylhydantoin to human and bovine albumins. Their results with this large group of drugs are precisely what one would have predicted from the methyl orange data (30, 133). None of these drugs was displaced until the molar ratio of FFA to albumin exceeded 3.5. These findings together with Goodman's studies have led to the view that oscillations in the plasma FFA concentration within the ordinary physiological range have no important influence on drug binding. On the other hand, extremely high FFA concentrations, such as those that occur after injection of heparin, are thought to reduce drug binding.

Our own very limited studies in this area are in only partial agreement with this commonly held view. As expected, we found that halofenate binding to human albumin was not reduced until 3 or more moles of palmitate was added (45). By contrast, clofibrate binding was reduced even when only 1–2 moles of palmitate was added (45). This, together with the ANS fluorescence results (38), suggests that the molecular interaction between albumin and a drug *is* influenced by the amount of FFA that is bound at the primary sites. With most drugs, however, the resulting binding change is too small to be established conclusively by equilibrium dialysis measurements.

Physiological metabolites

The possibility that changes in the plasma FFA concentration may influence the transport of a physiologically important substance has been examined in four cases: thyroxine, bilirubin, taurocholate, and tryptophan. Albumin has one strong binding site for thyroxine, the k_1 being about 2×10^6 M^{-1} (122, 135). Albumin also contains five or six weaker sites that have an average k_i for thyroxine of 6×10^4 M^{-1}. FFA displace thyroxine from human albumin, the order being oleate > linoleate > palmitate > laurate > octanoate (136). With palmitate, some displacement of thyroxine occurred even when the molar ratio of fatty acid to albumin was only 1.0. At a molar ratio of 3.1, a large decrease in thyroxine binding occurred. Clinical studies have revealed, however, that the usual physiological oscillations in FFA concentration do not alter thyroxine or triiodothyronine binding in the intact plasma of euthyroid subjects (137). The difference between this observation and what might be predicted from the albumin binding results is probably due to the fact that most of the circulating thyroid hormones are carried by thyroxine-binding globulin and prealbumin, not albumin. FFA appears to displace thyroid hormone slightly in thyrotoxicosis (137), perhaps because the "excess" hormone is bound to some extent by albumin.

Unlike thyroxine, taurocholate binding to albumin was not reduced when oleate was added (121). When large quantities of oleate were added, the amount of taurocholate that could be bound by albumin actually increased. Bilirubin also was not displaced from albumin by FFA, at least until more than 4–5 moles of FFA was added (138, 139). Spectrophotometric studies, however, indicated that the presence of FFA changed the molecular interaction between bilirubin and the albumin binding sites (138). This is consistent with my suggestion that the addition of even small amounts of FFA will influence the combination of albumin with a second ligand at the molecular level.

Changes in the plasma FFA concentration are thought to influence tryptophan transport. Albumin has a single strong binding site for tryptophan, and small amounts of oleate will displace tryptophan from this site (140, 141). Displacement also occurs in intact plasma, and this has led to the view that changes in the plasma FFA concentration control the availability of tryptophan to the brain (142, 143). It has been suggested that this, in turn, controls the production of the neurotransmitter serotonin.

Effect of organic compounds on fatty acid binding

Thorp (144) has suggested that pharmacological concentrations of clofibrate reduce the binding of FFA to plasma albumin. Subsequently, we demonstrated that uptake of palmitate and oleate by Ehrlich ascites cells increased as the clofibrate concentration was raised (145). Although the mechanism of this observation has not been elucidated, a likely explanation is that clofibrate weakens the binding of FFA to albumin, thereby making the fatty acid more available for uptake. Salicylates in high concentrations also reduce FFA binding to albumin (123). In spite of these potentially important findings, the general question of whether the presence of a second organic lig-

and will influence the binding of FFA to albumin has not been explored in any depth. Except in the case of bilirubin (138), most organic compounds are bound to albumin much less tightly than the 16 and 18 carbon atom fatty acids. These compounds would not be able to compete effectively with long-chain FFA for albumin binding sites as long as the molar ratio of FFA to albumin remains in the usual physiological range ($\bar{\nu}$ = 0.5–2.0). Any displacement of FFA by another organic ligand under these conditions almost certainly would have to result from either steric hindrance or a ligand-induced conformational change in the strong FFA binding sites. In light of the observations with clofibrate and salicylate, these possibilities merit further consideration.

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