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Effects of Fatty Acids on the Interaction of 1-Anilino-8-naphthalenesulfonate with Human Plasma Albumin

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SUMMARY

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The fluorescence of 1-anilino-8-naphthalenesulfonate (ANS) bound to human plasma albumin was enhanced when up to 2 moles of palmitate were added. Larger amounts of palmitate produced a progressive reduction in ANS fluorescene. Saturated and unsaturated fatty acids containing 14–18 carbon atoms produced similar biphasic effects on ANS fluorescence with human albumin. Fatty acids containing 10–12 carbon atoms produced only a reduction in ANS fluorescence, and those containing 2–8 carbon atoms had no effect. Similar results were obtained with canine albumin, whereas with bovine and rabbit albumins, fatty acids in all concentrations reduced ANS fluorescence. The tryptophan fluorescence of human albumin was decreased markedly by ANS, but not by palmitate. However, palmitate partially protected the tryptophan fluorescence against quenching by potassium iodide. Equilibrium dialysis measurements indicated that ANS binding to human albumin was reduced when 3 or more moles of long-chain fatty acid were added per mole of albumin, suggesting that the decrease in ANS fluorescence produced by high concentrations of fatty acid was due to displacement of ANS from albumin. ANS binding was essentially unchanged when 1 or 2 moles of fatty acid were present. Therefore the enhancement in ANS fluorescence produced by low concentrations of fatty acid appears to result from a change in the interaction of ANS with albumin rather than from an increase in the amount of ANS that is bound The data obtained with this fluorescent model compound suggest that the binding of some drugs to albumin may be influenced by changes in the plasma free fatty acid concentration. Small increases, such as those which occur under ordinary physiological conditions, are likely to alter the molecular interactions between albumin and the drug without appreciably affecting the strength of drug binding. When the molar ratio of fatty acid to albumin approaches 3, however, a decrease in drug binding is likely to occur.

INTRODUCTION

Plasma albumin serves as the carrier for a number of substances that are poorly soluble in aqueous media but must be transported through the blood. One of the most important of these is free fatty acid, the form in which fat is released from the adipose tissue for utilization elsewhere in the body (1, 2). The plasma free fatty acid concentration varies considerably during the course of a day, responding rapidly to changes in nutrition, physical activity, and environmental

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stimuli (3–6). Under ordinary conditions, the molar ratio of free fatty acid to albumin in human plasma varies between 0.5 and 2.0 (7). In a few situations, however, such as after vigorous physical exercise, molar ratios of 4.0 or more have been observed (3, 8). In addition to free fatty acid, human plasma albumin serves as the transport vehicle for a variety of commonly prescribed drugs. Therefore it is important to determine whether changes in free fatty acid concentration will influence the interaction of this protein with a drug.

The generally held view is that physiological changes in the plasma free fatty acid concentration have virtually no effect on drug transport by human plasma albumin. This concept originates from Goodman's observation (9) that methyl orange binding to human albumin was unaltered by the presence of up to 2 moles of fatty acid. The equilibrium dialysis studies of Rudman *et al.* (10) supported this view, for drug binding to albumin was unchanged until more than 3.5 moles of fatty acid were added. Certain of our studies with hydroxyphenylazobenzoate also support this interpreparation (11). Using equilibrium dialysis, we observed no significant change in hydroxyphenylazobenzoate binding to human albumin until 3 moles of fatty acid were added. However, an increase in cellular uptake of hydroxyphenylazobenzoate was noted when only 2 moles of fatty acid were present (11). Some additional questions were raised by fluorescence studies with bovine plasma albumin and 1-anilino-8-naphthalenesulfonate, a model ligand whose binding can be monitored by sensitive spectrophotofluorometric techniques (12). Addition of 1 or 2 moles of long-chain fatty acids perturbed ANS[2] fluorescence even though binding to bovine albumin, as measured by equilibrium dialysis, was not changed significantly (13).

The ANS findings suggested that even small changes in free fatty acid concentration might influence the interaction of an organic ligand with the secondary binding sites of albumin, at least at the molecular level. Before attempting to extend these observations to drug binding, however, we considered it important to determine whether the fatty acid effects on ANS fluorescence also occurred with albumins other than bovine plasma albumin. This was thought to be necessary because of the many differences that are known to exist between albumins from various species (14–17). Therefore we have extended the ANS fluorescence studies, placing particular emphasis on human albumin because of the need for precisely defining the effect of free fatty acids in man.

[2] The abbreviation used is: ANS, 1-anilino-8-naphthalenesulfonate.

METHODS

The serum albumins, obtained from Miles Laboratories, were treated with charcoal to remove inherent fatty acids, and then dialyzed (18, 19). ANS, purchased from Eastman Kodak, was recrystallized from water and dried at 110° for 8 hr (20). The protein solutions were made up to contain 0.05 M sodium phosphate and 0.1 M NaCl, pH 7.4. Protein concentration was determined by the biuret method (21). Fatty acids of 99% or greater purity were purchased from either Applied Science Laboratories or the Hormel Institute. The fatty acid–albumin complexes were prepared by incubating solutions of the protein with fatty acid-coated Celite (22). Fatty acid concentration was determined by titration (23). Palmitate solutions containing no protein were prepared by dissolving a measured quantity of sodium palmitate in warm H_2O and adding this dropwise to a large volume of buffered salt solution that was being stirred mechanically. Using this method, optically clear solutions were obtained as long as the final sodium palmitate concentration did not exceed 25 μM.

Fluorescence measurements were made using a Hitachi Perkin-Elmer model MPF-2A fluorescence spectrophotometer equipped with a recorder. A thermostatically controlled Lauda K-2R water circulator maintained the temperature of the cell holder at 25°. The ratio recording mode of operation was used to eliminate fluctuations of the source output. The fluorescent emission that was obtained as soon as the solution

was prepared did not change over the course of 6–8 hr. In most cases, however, the fluorescence measurements were made about 1 hr after the solutions were prepared. Fatty acids always were added to the albumin before ANS was added. The albumin concentration was 5 μM in most of the fluorescence experiments. Excitation and emission slit widths were set at 4 nm, and the light path of the cuvette was 1 cm. An excitation wavelength of 380 nm was used for the ANS experiments, and the fluorescent emission was recorded between 400 and 600 nm. For intrinsic fluorescence studies, excitation wavelengths of either 280 or 295 nm were used, and the emission was recorded between 260 and 380 nm.

ANS binding to human albumin was measured by equilibrium dialysis (11). The dialysis cells and membranes were purchased from Bel-Art Products, Pequannock, N. Y. (model 250). Before use, the membranes were placed in boiling distilled water and then soaked for 48 hr in fresh distilled water. Preliminary incubations at pH 7.4 demonstrated that neither crystalline human albumin nor [1-^{14}C]palmitate passed through these membranes when treated in this manner. By contrast, ANS equibrated across the membrane within 8–12 hr at 37°. Preliminary incubations also indicated that less than 2% of the ANS was bound to the membrane during these incubations. Twelve dialysis cells were used in each experiment. Exactly 1 ml of the 100 μM albumin solution was added to the left compartment. In the right compartment, 1 ml of the same sodium phosphate-NaCl buffer solution containing various amounts of ANS was added. Incubation was carried out for 18 hr at 37° in a temperature-controlled water bath with shaking at 20 oscillations/minute. Following incubation, the ANS concentration in the protein-free compartment was measured spectrophotometrically and, after correction for adsorption to the membrane, the concentrations of free and bound ANS in the albumin solution were calculated. The pH of the solutions on both sides of the membrane remained identical during incubation, indicating that corrections for the Donnan effect were not necessary.

RESULTS

ANS fluorescence. As shown in Fig. 1, the fluorescence spectra of 25 μM ANS bound to 5 μM plasma albumin differed somewhat, depending on the species from which the albumin was obtained. When excited at 380 nm, the relative maximum fluorescence intensities in the absence of fatty acids (dashed curves) were: bovine, 89; human, 72; rabbit, 55; canine, 42. The wavelength of maximum fluorescence of the ANS-albumin complexes varied only slightly: bovine, 469 nm; human, 466 nm; rabbit, 470 nm; canine, 468 nm. Two general types of responses were observed when palmitate was added (solid curves). With the bovine and rabbit albumins, all concentrations of palmitate produced a reduction in ANS fluorescence. The magnitude of the decrease became greater as the palmitate concentration was raised. By contrast, with the human and canine albumins, low concentrations of palmitate produced an *enhancement* in ANS fluorescence. At higher palmitate concentrations, however, ANS fluorescence was reduced. Palmitate did not alter the shape of the ANS emission spectrum or the wavelength of maximum fluorescence with any of the albumins.

From previous measurements of [1-^{14}C] palmitate binding to plasma albumin (19), we estimate that the unbound palmitate concentrations in the above experiments varied from 0.01 to 20 μM. These palmitate concentrations are too low for either micelles or gross aggregates to form, but they are in the range where dimerization may occur (24, 25). However, fluorescence changes were not observed when ANS was added to palmitate solutions in this concentration range. Therefore the fluorescence effects noted in the above experiments, in which the media contained albumin, almost certainly were not caused by interactions of ANS with unbound palmitate.

The effects of other physiologically important fatty acids on the interaction of ANS with bovine albumin already have been studied in detail (13). We wished to compare these results with those obtained using an albumin in which palmitate produced the *biphasic* ANS fluorescence effect. Be-

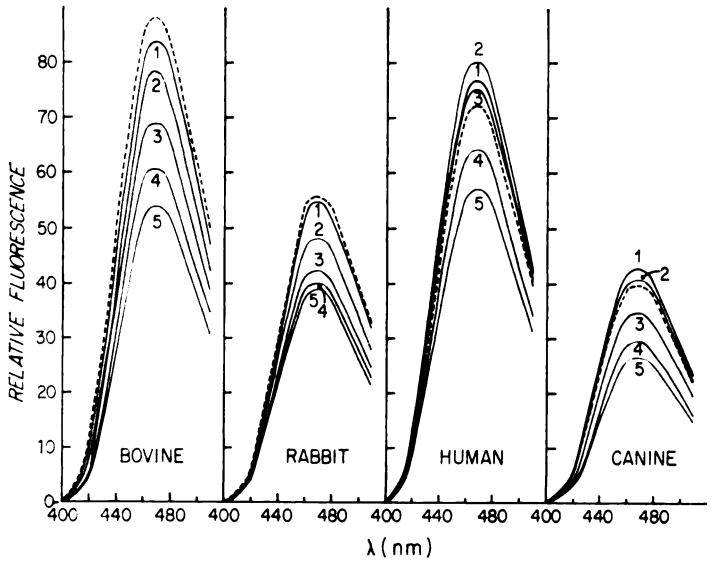

FIG. 1. *Effect of palmitate on ANS fluorescence spectrum with bovine, rabbit, human, and canine plasma albumins*

Measurements were made at 25° in a medium containing 0.1 M NaCl, 0.05 M sodium phosphate (pH 7.4), 5 μM albumin, and 2.5 μM ANS. The wavelength of excitation was 380 nm. In each case the dashed curve represents the spectrum obtained with fatty acid-free albumin. The solid curves represent spectra obtained in the presence of various amounts of palmitate, the number associated with each solid curve giving the molar ratio of total palmitate to albumin at which the particular spectrum was obtained.

cause of its importance, human albumin was selected for these additional studies. Figure 2 illustrates the effects of several medium- and long-chain saturated fatty acids on the fluorescence of ANS bound to human albumin. Fatty acids containing 2–6 carbon atoms had no effect, and the results with these acids are not shown. Octanoate also had little effect. Decanoate produced moderate reductions in ANS fluorescence when more than 1 mole was present, whereas laurate produced a reduction in all of the concentrations that were tested. Myristate, however, enhanced ANS fluorescence when only 1 mole was present, but it produced a reduction when larger amounts were added. Palmitate enhanced ANS fluorescence when up to 2 moles were added, whereas larger amounts produced a reduction. As seen in Fig. 3, the effect of stearate was similar to that of palmitate. Figure 3 also shows that the degree of unsaturation had relatively little effect on the changes in ANS fluorescence produced by fatty acids containing 18 carbon atoms All these acids enhanced ANS fluorescence when present in low concentrations, and they reduced fluorescence when added in higher concentrations. The amount of enhancement produced by linolenate, however, was somewhat smaller than that produced by the other 18-carbon atom acids. The complete emission spectrum obtained witb each of these fatty acids indicated that, like palmitate, they produced no alteration in the wavelength of maximum ANS fluorescence.

The enhancement of ANS fluorescence produced by low concentrations of palmitate occurred only in the pH range 6–9, whereas the reduction in fluorescence produced by higher palmitate concentrations cocurred over a wider pH range, 6–11. Studies on the effects of ANS concentration revealed that with 5 μM albumin the decrease in ANS fluorescence produced by 25 μM palmitate was maximal between 15 and 50 μM ANS. The enhancement in ANS fluorescence produced by 10 μM palmitate was maximal over a smaller concentration range, 10–25 μM ANS.

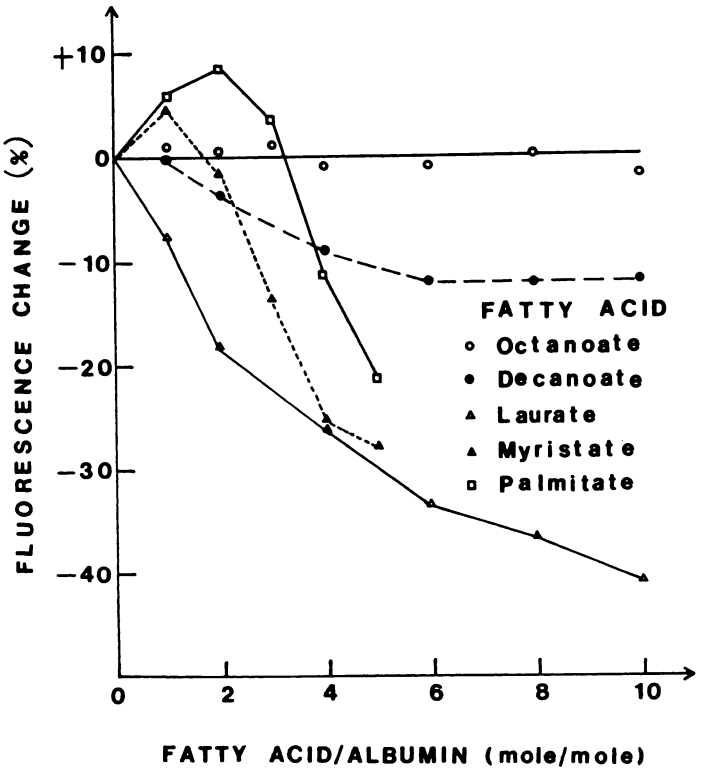

FIG. 2. *Effect of saturated fatty acids of varying chain lengths on ANS fluorescence with human plasma albumin*

The conditions were the same as those described in Fig. 1, but only the relative fluorescence intensities at 466 nm are shown.

Intrinsic fluorescence. The effects of ANS on the ultraviolet fluorescence of human plasma albumin are listed in Table 1. Fluorescence was decreased markedly as ANS was added, and this was associated with a large blue shift in the wavelength of maximum fluorescence. When 1 mole of ANS was present the tryptophan fluorescence was decreased by more than 95%. Addition of 1–3 moles of palmitate had essentially no effect on either the degree of quenching or the magnitude of the blue shift produced by ANS. In the absence of ANS, addition of palmitate produced no appreciable change in the magnitude of fluorescence and only a very small blue shift in the wavelength of maximum fluorescence.

As seen in Table 2, the presence of KI reduced the tryptophan fluorescence of human albumin. Addition of increasing amounts of palmitate decreased the KI-induced quenching. This occurred in concentrations at which palmitate itself did not enhance the tryptophan fluorescence.

Equilibrium dialysis studies. The effects of oleate and palmitate on ANS binding to human albumin were measured by equilibrium dialysis. These data were analyzed by the method of Scatchard (26), in which $\bar{\nu}$ represents the molar ratio of bound fatty acid to albumin and c represents the unbound ANS concentration in molarity. Figure 4 illustrates the effects of oleate on ANS binding. A considerable reduction in ANS binding was noted when 4 moles of oleate were added. The changes produced by 2 moles of oleate, however, were so small as to be of questionable significance. Therefore, in order to obtain better quantitative estimates of the effect of small amounts of fatty acid, a series of equilibrium dialysis experiments were performed, using prepara-

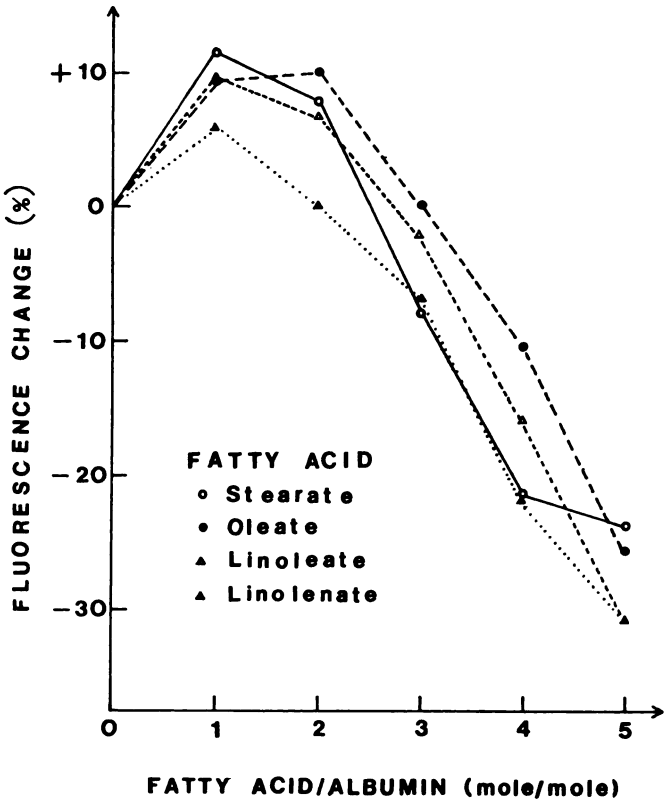

FIG. 3. *Effect of fatty acid unsaturation on ANS fluorescence with human albumin*
The conditions were the same as those described in Fig. 2.

TABLE 1

Effect of ANS and palmitate on intrinsic fluorescence of human plasma albumin

The medium contained 0.1 M NaCl, 0.05 M Na_2HPO_4 (pH 7.4), and 50 μM human albumin. Excitation was at 280 nm, and the fluorescence spectra between 380 and 400 nm were recorded. Only the maximum relative fluorescence and the wavelength at which maximum fluorescence occurred are listed.

ANS	Relative fluorescence				λ_{max}			
	0[a]	1	2	3	0	1	2	3
μM	*units*				*nm*			
0	80	77	79	78	340	338	338	338
12.5	34	33	29	29	330	330	330	328
25.0	13	12	12	11	322	318	318	317
37.5	5.0	5.0	5.0	4.5	315	315	316	313
50.0	2.8	2.8	2.6	2.8	314	314	315	312

[a] Moles of palmitate per mole of albumin.

tions of human albumin that contained 0–4 moles of palmitate. Multiple determinations were made at four points along each binding isotherm, so that standard errors for these data points could be calculated. The results are listed in tabular form (Table 3) because there was considerable overlap in portions of the data, making a composite graphical display appear cluttered. As predicted by the oleate experiments, ANS binding was reduced significantly at all points when 3 or 4 moles of palmitate were added ($p < 0.01$). When 1 or 2 moles of palmitate were present, a tendency toward decreased ANS binding was noted. In these cases, however, statistical comparisons revealed that only the values of $\bar{\nu}/c$ obtained at the highest amount of bound ANS, 4 moles/mole of albumin, were significantly reduced ($p < 0.01$).

The ANS binding data were analyzed in

TABLE 2

Effect of palmitate on iodide quenching of intrinsic fluorescence of human albumin

The medium contained 10 μM human albumin, 0.05 M Na_2HPO_4, the amounts of KI listed, and varying amounts of NaCl so that the ionic strength was 0.4 in each case. The final pH was 7.0. Excitation occurred at 295 nm, and the fluorescence intensity was recorded between 300 and 400 nm. Only the fluorescence intensity at 340 nm is shown.

KI	Relative fluorescence			
	0[a]	1	2	3
M	*units*			
0	80	81	79	79
0.05	52	54	57	63
0.10	44	46	51	58
0.20	38	40	45	53

[a] Moles of palmitate per mole of albumin.

terms of two independent classes of albumin binding sites (26). In the palmitate experiments, the parameters calculated for fatty acid-free albumin are $n_1 = 3$, $k'_1 = 8 \times 10^4$ M^{-1}, $n_2 = 8$, and $k'_2 = 2.5 \times 10^3$ M^{-1}, where n_i is the number of binding sites in the ith class (to the nearest integer value) and k'_i is the average apparent association constant for the ith class. These values apply only to the albumin preparation used for the palmitate experiments; the one used in the oleate experiments, as seen by inspection of Fig. 4, gave slightly higher values for the binding parameters. As the palmitate concentration was raised, n_1 did not change appreciably, but k'_1 decreased, the values being 7.3×10^4 M^{-1} with 1 mole of palmitate, 7.0×10^4 M^{-1} with 2 moles, 6.2×10^4 M^{-1} with 3 moles, and 5.2×10^4 M^{-1} with 4 moles. Since the actual data points listed in Table 3 are not significantly different from the control values when 1 or 2 moles of palmitate were present, the apparent decreases in the k'_1 values in these cases probably are not real. The range of the ANS binding data was insufficient to make meaningful comparisons between the various n_2 and k'_2 values.

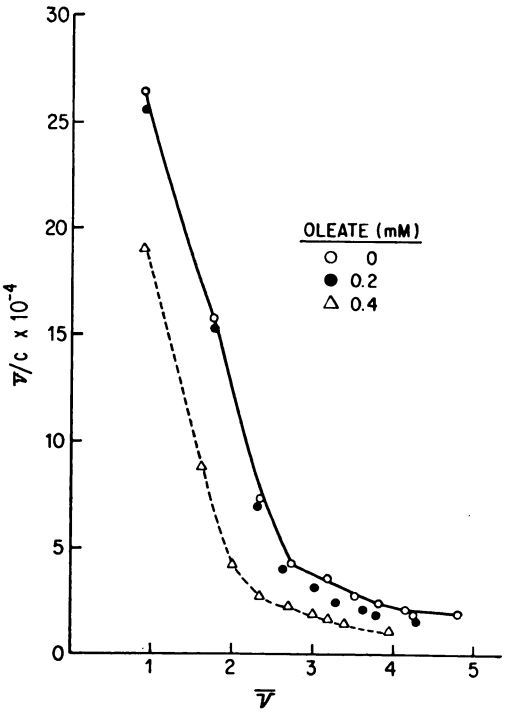

FIG. 4. *Scatchard plot of binding of ANS to human plasma albumin*

The effect of 2 and 4 moles of oleate on ANS binding is shown. Incubation was done at 37°. The medium contained 0.1 M NaCl, 0.05 M Na_2HPO_4 (pH 7.4), and 0.1 mM albumin.

DISCUSSION

The equilibrium dialysis data indicate that the decrease in ANS fluorescence produced by 3 or more moles of long chain fatty acids per mole of albumin is due to a reduction in ANS binding to albumin. Like ANS, the binding of a number of drugs to human albumin also is reduced when more than 3 moles of fatty acid are added (10). In an attempt to determine the mechanism of the decrease in ANS binding, the data presented in Fig. 4 and Table 3 were plotted in double-reciprocal form, $1/\bar{\nu}$ against $1/c$. However, as predicted by the fact that two slopes were observed on the Scatchard plots (Fig. 4), each of the double-reciprocal curves also contained two slopes. Moreover, the five double-reciprocal curves obtained from the data in Table 3 did not intersect at a common point. Therefore the displacement of ANS from human albumin by 3 or more moles of palmitate cannot be explained on the basis of competitive binding. An alternative explanation is suggested by the spec-

TABLE 3

Effect of palmitate on ANS binding to human albumin

These measurements were made by equilibrium dialysis at 37° in a medium containing 0.1 M NaCl, 0.05 M sodium phosphate (pH 7.4), and 1×10^{-4} M human albumin. Each value is the mean ± standard error of four determinations.

ANS:albumin molar ratio, $\bar{\nu}$	$\bar{\nu}/c \times 10^{-5}$				
	0[a]	1	2	3	4
0.9	19.1 ± 1.2	17.6 ± 2.1	16.4 ± 1.5	13.9 ± 0.6[b]	12.9 ± 0.6[b]
1.7	13.8 ± 0.1	13.6 ± 0.1	14.2 ± 0.1	12.0 ± 0.08[b]	9.9 ± 0.06[b]
2.8	4.6 ± 0.07	4.5 ± 0.07	4.5 ± 0.07	3.3 ± 0.08[b]	3.1 ± 0.07[b]
4.0	2.3 ± 0.03	2.1 ± 0.02[b]	2.0 ± 0.02[b]	1.6 ± 0.01[b]	1.3 ± 0.01[b]

[a] Moles of palmitate per mole of albumin.

[b] Significantly different from the data obtained in the absence of fatty acid ($p < 0.01$).

troscopic studies of Steinhardt *et al.* (27). These investigators found that the initial tyrosine red shifts produced by *n*-alkyl ligands were reversed to blue shifts as the ligand concentration was raised. This reversal occurred at much lower molar ratios of ligand to albumin than those required to produce massive unfolding of the protein. Steinhardt *et al.* attributed the spectroscopic reversal to exposure of previously buried tyrosine residues, i.e., a microdisorganization of the albumin tertiary structure. We suggest that the secondary binding sites of albumin are progressively altered as the microdisorganization increases and that this may be the cause of the observed decrease in ANS binding.

Another conclusion from the equilibrium dialysis experiments is that the enhancement of ANS fluorescence produced by 1 or 2 moles of long-chain fatty acid is *not* due to any increase in ANS binding. Therefore, at least in some cases, the magnitude of fluorescence can change as a result of other factors besides a change in the number of ligands bound to the protein. This indicates that fluorescence measurements cannot always be used as a quantitative estimate of ligand binding. Studies with 2-*p*-toluidinylnaphthalene-6-sulfonate and model peptides lead to a similar conclusion, for they also demonstrate that a fluorescence change is not necessarily associated with a change in the amount that is bound (28). Some insight into the probable mechanism of the fluorescence enhancement is provided by the intrinsic fluorescence measurements. The data in Table 1 indicate that ANS binds in the region of the lone tryptophan residue of human albumin. Spectroscopic studies indicate that steroids also bind to this region (29), and it is generally accepted that tryptophan forms a part of an important nonpolar binding site of human albumin (30). As opposed to ANS, palmitate in low concentrations did not alter the tryptophan fluorescence (Table 1). Taken together, these findings suggest that the first 2 moles of long-chain fatty acid do not bind to the same sites as ANS. It may be argued, however, that ANS produces quenching through energy transfer and that this could not occur with fatty acids even if they bound in the same region. Yet with bovine albumin fatty acids and their derivatives, like ANS (12), quench the tryptophan fluorescence (31, 32). Moreover, the spectroscopic findings of Steinhardt *et al.* (27) also indicate that the first 2 moles of fatty acid derivatives do not perturb the tryptophan spectrum of human albumin; instead, they perturb the tyrosine spectrum. This supports the view that the first 2 moles of long-chain alkyl derivatives do not bind to the region of human albumin that contains the lone tryptophan residue.

The interpretation that the first 2 moles of palmitate do not bind to the same sites as ANS is consistent with the human albumin binding model proposed by Goodman (9), in which the two primary binding sites are considered to be accessible to long-chain fatty acids but not to bulky organic ligands.

Studies of fatty acid effects on the tyrosine absorption spectrum of human albumin also are consistent with the Goodman model (33). If this view is correct, how do long-chain fatty acids produce the enhancement in ANS fluorescence? A possible explanation is suggested by the results with KI. Palmitate protects against quenching of the tryptophan fluorescence by KI (Table 2) although it does not bind to the site containing tryptophan (Table 1). This suggests that the tryptophan site is affected when palmitate combines with other regions of the albumin molecule, through either steric hindrance or a conformational change. One might expect that molecular interactions between an organic ligand and the tryptophan site would be altered as a result of this. In the case of ANS, this is manifested as fluorescence enhancement without any marked change in binding affinity. It is somewhat surprising that no change in the wavelength of maximum ANS fluorescence accompanied the postulated increase in quantum yield. A possible explanation is that palmitate binding partially removed a protein-quenching group, such as a carboxylate ion, from the ANS environment in such a way that the polarity of the ANS binding site was not changed appreciably.

The fact that, unlike palmitate, 1 or 2 moles of laurate and decanoate reduced ANS fluorescence also is consistent with the human albumin binding model proposed by Goodman (9). According to Goodman's interpretation, medium-chain fatty acids cannot interact with the primary binding sites. Instead, they bind to the same secondary sites as ANS. Therefore one might expect some displacement of ANS even when only 1 or 2 moles of laurate or decanoate are added, because of competition for the same binding sites. By contrast, no ANS displacement would be produced by 1 or 2 moles of palmitate, because it would be bound for the most part to the primary sites.

In conclusion, we recognize the possibility that data obtained with a model fluorescent ligand may have no application to drug binding. On structural grounds, however, it is reasonable to assume that ANS might be a suitable experimental model for binding studies. Moreover, we have presented preliminary evidence that the human albumin binding sites for chlorophenoxyisobutyrate, a commonly used hypolipidemic drug, are the same as those for ANS (34). Therefore we believe that some cautious extrapolation of the present results to drug binding is justified. The results with ANS actually are compatible with many of the current ideas concerning the role of free fatty acids in drug transport (10). They support the view that major quantitative changes in drug binding do not occur when the molar ratio of fatty acid to albumin is less than 2. They also support the view that reductions in drug binding are likely to occur when the molar ratio rises and is between 2 and 4. Although the plasma free fatty acid concentration usually is below these levels, such values have been reported in man (3). The present results with ANS, however, are not entirely consistent with the prevalent view that 1 or 2 moles of fatty acid have absolutely no influence on the drug-binding sites of human albumin (9). Based upon the fluorescence measurements, it appears that small amounts of long-chain fatty acids will alter the molecular interactions between an organic ligand and the secondary albumin sites. Although the equilibrium dialysis measurements indicate that, in the case of ANS, these molecular alterations do not cause gross binding changes, the possibility that such alterations affect the transport of at least some drugs by albumin requires further consideration.

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