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Effect of Fatty Acids on the Binding of 1-Anilino-8-naphthalenesulfonate to Bovine Serum Albumin†

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ABSTRACT: The fluorescence of 1-anilino-8-naphthalenesulfonate (ANS) bound to bovine serum albumin was reduced by the presence of medium or long-chain fatty acids. This occurred over the pH range of 4–10. Quenching increased as the molar ratio of fatty acid to albumin was raised, the maximum reductions being in the range of 45–55%. The degree of quenching produced by a given amount of fatty acid was dependent upon the ANS–albumin molar ratio. Fatty acid induced quenching was not accompanied by any change in the wavelength of maximum fluorescence. Effects on ANS fluorescence similar to those noted with bovine albumin were observed when palmitate was added to human, rabbit, or equine albumins. Equilibrium dialysis studies revealed that ANS binding to bovine albumin was decreased when either palmitate or laurate were present. Therefore, the fatty acid induced quenching of ANS fluorescence probably was due to displacement of ANS from albumin binding sites. These results suggest that variations in the plasma free fatty acid concentration may be important in regulating the capacity of albumin to transport other nonpolar organic compounds.

Fluorescent probes such as 1-anilino-8-naphthalenesulfonate (ANS)[1] have been employed to examine the structure and interactions of proteins, particularly the nature of their nonpolar binding sites (Stryer, 1965; Turner and Brand, 1968). Bovine serum albumin has been studied extensively by this technique (Weber and Young, 1964; Daniel and Weber, 1966; Weber and Daniel, 1966), and albumin often has served as a model protein for structural interpretations.

A major physiological function of albumin is to transport long-chain free fatty acid in the plasma (Dole, 1956; Gordon and Cherkes, 1956), and the binding of fatty acids to albumin has been investigated in detail (Boyer *et al.*, 1946; Teresi and Luck, 1952; Goodman, 1958; Reynolds *et al.*, 1968; Spector *et al.*, 1969, 1971). In the course of studies with albumin, we found that addition of palmitate altered the fluorescence of albumin-bound ANS. We thought it of interest to explore this observation in order to better understand the effect of lipids on the binding of fluorescent probes to proteins and to gain insight into possible interactions between fatty acids and other nonpolar ligands that are transported by serum albumin.

Methods

ANS, obtained from Eastman Kodak Co., was recrystallized from water and then dried at 110° for 8 hr (Weber and Young, 1964). Serum albumins, purchased from Miles Laboratories, were treated with charcoal and then dialyzed to remove inherent fatty acids (Chen, 1967; Spector *et al.*, 1969). Sodium phosphate was added to the protein solutions so that the final concentration was 0.05 M (pH 7.4). Protein concentration was determined by the biuret method with dried, crystalline albumin as the standard (Gornall *et al.*, 1949). Fatty acids were purchased from either Applied Science Laboratories or the Hormel Institute and were of the highest purity available commercially (99%). Fatty acid–albumin complexes were prepared by adding slowly a warm solution of the sodium salt of the fatty acid to the albumin solution. The concentration of the fatty acid was determined by titration (Trout *et al.*, 1960).

Fluorescence measurements were made with a Hitachi Perkin-Elmer Model MPF-2A fluorescence spectrophotometer equipped with a recorder. A thermostatically controlled Lauda K-2/R water circulator (Brinkmann Instruments) maintained the temperature in the cell holder at 25°. The ratio recording mode was used to eliminate fluctuations of the source output. The excitation wavelength was 380 nm, and the fluorescent emission was recorded between 400 and 600 nm. Fluorescence measurements were taken at least 1 hr after the incubation mixtures were prepared.

ANS binding to albumin was measured at 37° by equilibrium dialysis (Spector and Imig, 1971). Equilibrium was attained in this system within 16 hr. Preliminary studies indi-

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[1] Abbreviation used is: ANS, 1-anilino-8-naphthalenesulfonate.

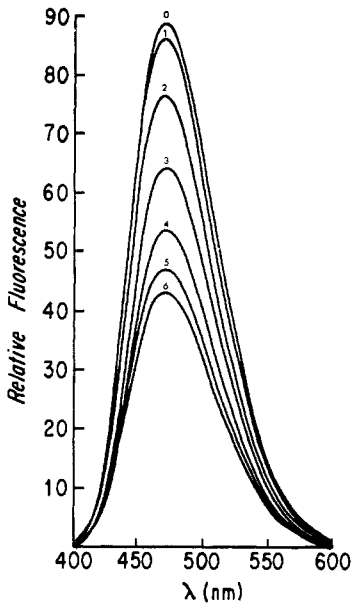

FIGURE 1: Fluorescence spectra of ANS–albumin complexes containing varying amounts of palmitate. The wavelength of excitation was 380 nm. These media contained 5×10^{-6} M albumin, 2.5×10^{-5} M ANS, and 0.05 M sodium phosphate buffer (pH, 7.4). The molar ratio of palmitate to albumin varied from 0 to 6 as is shown on the figure.

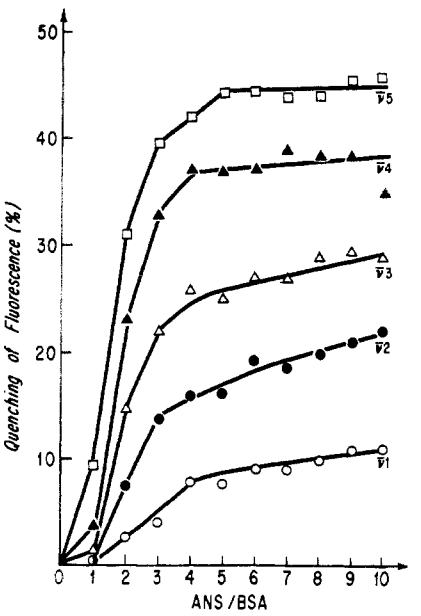

FIGURE 3: Effect of ANS concentration on the degree of quenching produced by palmitate at 470 nm. The molar ratio of palmitate to albumin, shown on the figure as $\bar{\nu}$, varied from 1 to 5. The conditions of incubation were the same as in Figure 1 except that the ANS concentration was varied from 5×10^{-6} to 5×10^{-5} M.

cate that neither albumin nor palmitate passed through the dialysis membrane under these conditions, and only negligible quantities of ANS were adsorbed on the membrane. The pH of the solutions on both sides of the dialysis membrane remained identical during incubation, indicating that no appreciable Donnan effect occurred in these binding experiments.

Results

Fluorescence of ANS–Albumin Complexes. Fluorescence spectra of ANS–albumin complexes excited at 380 nm are shown in Figure 1. In each case, the molar ratio of ANS to albumin was 5. Addition of palmitate produced increasing quenching of ANS fluorescence, but there was no change in the wavelength of maximum emission, 469 nm. Figure 2 illustrates the degree of quenching produced by palmitate or decanoate relative to the fatty acid:albumin molar ratio. In both cases,

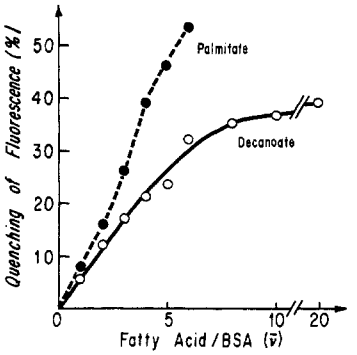

FIGURE 2: Effect of various concentrations of palmitate or decanoate on the fluorescence of the ANS–albumin complex at 470 nm. The conditions are the same as those listed in Figure 1.

there was an approximately linear increase in quenching as the molar ratio was raised from 1 to 6. At a given concentration, the amount of quenching produced by decanoate was considerably less than that produced by palmitate. Under these conditions, the maximum molar ratio that could be achieved with palmitate and other long-chain fatty acids was in the range of 5 to 6. With shorter chain length acids such as decanoate, optically clear solutions having fatty acid:albumin ratios of 20 could be prepared, enabling us to examine the effects of high fatty acid concentrations on ANS fluorescence. Little increase in quenching was observed when the decanoate:albumin ratio was raised from 8 to 20.

As shown in Figure 3, the degree of quenching produced by a given amount of palmitate was dependent upon the ANS concentration. When the ANS:albumin molar ratio was 1, quenching did not occur until more than 2 moles of palmitate was added, and 5 moles of palmitate produced only a 9% reduction in fluorescence. In contrast, quenching was produced by only 1 mole of palmitate when the ANS:albumin ratio was raised to 2, and 5 moles of palmitate produced a 31% reduction in ANS fluorescence. When 4 or 5 moles of palmitate was present, there was no increase in percentage quenching when the ANS:albumin ratio was raised above 5. However, with lesser amounts of palmitate, there was a small increase in percentage quenching when the ANS:albumin ratio was raised from 4 to 10.

The relationship of fatty acid structure to quenching of ANS fluorescence is presented in Table I. Complete spectra similar to those shown in Figure 1 were obtained with each acid, but only the major findings are listed in this table. Little quenching occurred with fatty acids containing less than eight carbon atoms. Octanoate and decanoate produced moderate quenching, whereas all of the long-chain acids, both saturated and unsaturated, were about as effective as palmitate. With each of the medium and long-chain acids, there was an increase in the degree of quenching produced by a given amount of acid when the ANS:albumin ratio was

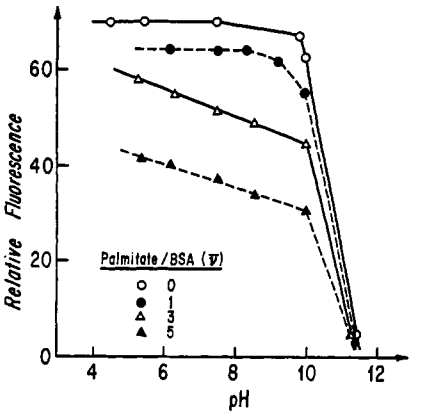

FIGURE 4: Effect of pH on ANS fluorescence and palmitate-induced quenching at 470 nm. The palmitate:albumin molar ratio is shown on the figure. The conditions were the same as in Figure 1.

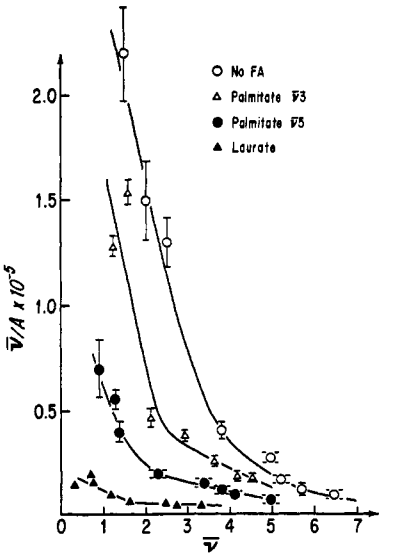

FIGURE 5: Effect of fatty acid on ANS binding to albumin. Equilibrium dialysis incubations were done at 37° in 0.05 M sodium phosphate (pH 7.4) for 16 hr. The points with fatty acid free albumin and palmitate–albumin ($\bar{\nu}$ = 5) are the mean values of five determinations. The points with palmitate–albumin ($\bar{\nu}$ = 3) are the mean values of three determinations. Standard errors are shown for all of these values. The laurate–albumin data ($\bar{\nu}$ = 8) are taken from a single experiment.

raised from 2 to 5, but no further increase occurred when the ratio was raised from 5 to 8. None of the acids produced any shift in the wavelength of maximum fluorescence. Methyl palmitate was less effective than the corresponding fatty acid, palmitate, and cetyl alcohol did not cause any quenching. However, the degree of quenching produced by dodecyl sulfate was almost the same as that produced by laurate. The method of addition of palmitate to albumin had little effect on the degree of quenching, for similar reductions occurred when palmitate was added to the protein by mixing with soap solution, by incubation with a Celite complex or by incubation with a heptane solution.

As shown in Figure 4, palmitate was effective in reducing the fluorescence of the ANS–albumin complex over a wide pH range. It was not possible to obtain readings below pH 4, for the albumin solutions containing fatty acid became opalescent at this point. The fluorescence intensity of the ANS–albumin complex decreased markedly when the pH exceeded 10, and palmitate did not produce any appreciable quenching at pH values of greater than 10.5.

As shown in Table II, palmitate also reduced the fluorescence of ANS in the presence of albumins other than bovine albumin. Quenching occurred with rabbit, equine, and human albumins, but the magnitude was less than with bovine albumin. There was no increase in quenching with these albumins when the ANS:protein ratio was raised from 2 to 6, a finding that differed from that made with bovine albumin.

Binding Studies. In an attempt to elucidate the mechanism of the fatty acid induced quenching of ANS fluorescence, the effects of fatty acid on ANS binding to albumin were investigated by equilibrium dialysis. As shown in Figure 5, a Scatchard plot demonstrated that fatty acid free albumin contained at least two classes of ANS binding sites. These data

TABLE I: Effect of Fatty Acid Structure on ANS Fluorescence.

Fatty Acids and Derivatives	Decrease in Fluorescence Intensity (%)					
	ANS: Albumin = 2		ANS: Albumin = 5		ANS: Albumin = 8	
	$\bar{\nu}$ = 2[a]	$\bar{\nu}$ = 5	$\bar{\nu}$ = 2	$\bar{\nu}$ = 5	$\bar{\nu}$ = 2	$\bar{\nu}$ = 5
Acetic	0	0	0	0	0	0
Hexanoic	2	4	0	0	0	0
Heptanoic	2	6	2	7	2	5
Octanoic	8	13	6	10	2	6
Decanoic	11	23	16	29	12	23
Lauric	17	31	22	39	18	35
Myristic	6	24	13	33	15	31
Palmitic	8	32	16	45	20	44
Stearic	8	32	16	40	14	37
Oleic	9	33	16	44	17	43
Linoleic	12	39	20	52	21	51
Methyl palmitate	2	*b*	10		9	
Cetyl alcohol	0	*b*	0		0	
Dodecyl sulfate	7	28	18	36	14	33

[a] $\bar{\nu}$, molar ratio of ligand to albumin. [b] It was not possible to prepare optically clear lipid–albumin solution of greater than molar ratio 2 with either methyl palmitate or cetyl alcohol.

TABLE II: Palmitate-Induced Quenching of ANS Fluorescence with Different Albumins.[a]

Serum Albumin	Decrease in Fluorescence Intensity (%)			
	ANS:Albumin = 2		ANS:Albumin = 6	
	$\bar{\nu}$ = 2[b]	$\bar{\nu}$ = 4	$\bar{\nu}$ = 2	$\bar{\nu}$ = 4
Bovine	9	29	17	39
Rabbit	7	25	8	22
Equine	3	17	3	16
Human	1	13	1	12

[a] Albumin concentration 5 × 10^{-6} M. [b] Molar ratio of palmitate to albumin.

were fitted adequately to a binding model having $n_1k_1' = 3.5 \times 10^5$ M^{-1} and $n_2k_2' = 1.2 \times 10^4$ M^{-1}. ANS binding was reduced by the presence of moderate amounts of palmitate or laurate. With palmitate at molar ratio 3, the ANS binding parameters were $n_1k_1' = 3.3 \times 10^5$ M^{-1} and $n_2k_2' = 10^4$ M^{-1}; and at molar ratio 5, they were $n_1k_1' = 10^5$ M^{-1} and $n_2k_2' = 6 \times 10^3$ M^{-1}. With laurate at molar ratio 8, the binding parameters were reduced further to $n_1k_1' = 2 \times 10^4$ M^{-1} and $n_2k_2' = 2 \times 10^3$ M^{-1}. Significant differences in ANS binding were not observed when palmitate–albumin of molar ratio 1 was compared with fatty acid free albumin, and the data obtained at molar ratio 1 have been omitted from this figure.

Discussion

The fluorescence of albumin-bound ANS was reduced when the protein contained medium or long-chain fatty acids. Most serum albumins, even in crystalline form, contain some free fatty acid (Kendall, 1941; Chen, 1967). Moreover, the fatty acid content of commercially available albumins varies, even in preparations obtained from a single species, *e.g.*, bovine (Chen, 1967). The present results suggest that albumin preparations which are used for quantitative studies involving fluorescent probes either should be freed of inherent fatty acids or should have their fatty acid content and composition accurately determined. Addition of oleic acid has been observed to reduce the fluorescence of ANS bound to submitochondrial particles (Harris, 1971). Therefore, some attention also should be given to the free fatty acid content when substances other than albumin are investigated with fluorescent probes, particularly membrane or enzyme preparations that may be in contact with fatty acid *in vivo* or in which free fatty acid may be generated during isolation.

Equilibrium dialysis studies indicated that the decrease in fluorescence was associated with reduced ANS binding to albumin. Competition between fatty acids and trinitrobenzenesulfonate for the same albumin binding sites has been observed (Anderson *et al.*, 1971). On the other hand, the data in Figure 3 are not compatible with a simple competitive binding mechanism. Addition of large quantities of ANS to albumin containing 1 or 2 moles of palmitate did not reduce the degree of quenching, suggesting that ANS does not compete effectively with palmitate for the strong albumin binding sites. This finding is compatible with the fact that the parameters computed for ANS binding to albumin are 50 to 100 times smaller than those computed previously for palmitate binding to albumin (Spector *et al.*, 1969). Furthermore, addition of 1 mole of palmitate affected the fluorescence intensity of more than a single mole of ANS (Figure 3). A similar effect of fatty acids was noted with albumin-bound methyl orange (Cogin and Davis, 1951). In this case, the presence of fatty acid at one binding site sterically hindered the combination of methyl orange with several adjacent binding sites. A similar mechanism would explain the effects of small amounts of fatty acid on the fluorescence of several moles of bound ANS. Alternatively, the possibility that fatty acid binding produced structural changes in albumin which altered the interaction of the remaining sites with ANS cannot be excluded. Small changes in protein structure have been noted when large organic compounds bind to albumin (Markus and Karush, 1958).

In addition to serving as a carrier for free fatty acid and other metabolites, serum albumin transports many commonly used drugs (Sellers and Koch-Weser, 1971). Like ANS, most of these drugs are nonpolar aromatic or heterocyclic compounds. Therefore, a sensitive fluorescent probe such as ANS might be a useful model for investigating certain aspects of the interaction of these compounds with albumin. Equilibrium dialysis studies have shown that free fatty acids will displace many drugs from serum albumin (Rudman *et al.*, 1971). However, because of experimental variability, significant changes were detected by equilibrium dialysis only when the fatty acid:albumin molar ratio was 3.5 or greater. Molar ratios of this magnitude seldom occur physiologically (Havel *et al.*, 1967). On the other hand, the present fluorescence measurements using ANS as a model indicate that variations in the free fatty acid:albumin molar ratio within the usual physiological range also may alter the binding capacity of albumin. This finding suggests that even relatively small changes in the plasma free fatty acid concentration may be important in regulating the transport of other albumin-bound organic ligands.

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