Drug Interactions. VII.[1] The Fatty Acid Binding Properties of Bovine Serum Albumin

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The interrelationships between fatty acid and probe binding to bovine serum albumin (BSA) were investigated by a fluorescence method, using 1-anilinonaphthalene-8-sulfonate (ANS) and N-phenyl-1-naphthylamine (NPN) as fluorescent probes. The fatty acid-induced quenching of probe fluorescence is probably due to the displacement of probes from BSA binding sites. It appears that NPN and ANS are bound to BSA at the same sites. Various molar ratios of capric acid, lauric acid and palmitic acid were added to defatted BSA in the presence of ANS, and the association constants of the fatty acids were calculated. Measurements of ANS fluorescence (λ_{max} 465 nm) and the fluorescence (λ_{max} 340 nm) of the ANS-BSA complex as well as the circular dichroism spectra of BSA in the presence of fatty acids in pH 7.4 phosphate buffer indicated that fatty acids induce different conformational states of the albumin molecule. One of the strongest binding sites of long-chain fatty acids may be the site which is labeled by TNBS (2,4,6-trinitrobenzene-sulfonic acid), located in the carboxyl-terminal half of the molecule, which does not contain tryptophan residues. It is suggested that the mechanism of inhibition of the probe-BSA binding by capric acid may involve competition for the same binding sites. In contrast, the decreases in probe binding with BSA in the presence of lauric acid, palmitic acid or stearic acid are due not only to a displacement of the probes by fatty acids from the probe-binding sites but also to a simultaneous conformational change of the probe-binding sites caused by the interaction of the probes and fatty acids.

Keywords——drug interactions; fatty acid; bovine serum albumin; binding and displacement; ANS and NPN; fluorescence titration; CD measurements; partition experiment; iodoacetamide treatment of BSA; TNBS treatment of BSA

A major physiological function of albumin is to transport both endogenous (*e.g.* bilirubin, tryptophan, hemin, and fatty acids) and exogenous (*e.g.* certain drugs and dyes) substances in the plasma. Many studies on the binding of these substances to plasma albumin have been reported.[3] A review of the available information on fatty acid binding to plasma albumin has been published by Spector.[4] The effect of free fatty acids on the binding of various drugs to albumin is a subject of continuing interest[5] because of the serious clinical implications.[6] In a previous report we have also described the competitive inhibition of the binding of thiopental or chlorpropamide to serum albumin by lauric acid.[7] Santos and

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Spector have shown that fatty acid-induced quenching of 1-anilinonaphthalene-8-sulfonate (ANS) fluorescence is probably due to the displacement of ANS from binding sites on albumin.[8] An inhibition of drug-protein binding by fatty acids due to conformational changes in the drug-binding sites of albumin in the presence of fatty acids have also been suggested,[5c,d] though no direct experimental evidence of such conformational changes in the protein structure is available. Fuller and Hunter suggested that the binding sites for fatty acids are located near the single sulfhydryl group, and that oxidation of this sulfhydryl group alters the binding of fatty acids at this site.[9] On the other hand, Reed *et al.* showed that the three strongest sites for the binding of palmitic acid are located in the carboxyl-terminal two-thirds of the molecule of BSA.[10] We have demonstrated that fluorescent probes such as ANS and N-phenyl-1-naphthylamine (NPN) are useful to study the binding of small molecules to BSA.[11] This work was conducted to study the mechanism of binding of various fatty acids to BSA, using the fluorescent probe technique.

Experimental

Materials——Bovine serum albumin (BSA) Fraction V (Wako Pure Chemical Industries, Ltd.) was used in this study, and its molecular weight was assumed to be 69000. BSA was treated with charcoal and then dialyzed to remove intrinsic fatty acids.[12] The fatty acid content of BSA, as measured by titration with NaOH using Nile Blue A as an indicator,[12] was less than 0.2 mol of fatty acid per mole of albumin. The concentrations of albumin solutions were determined from the absorbance of the peak at 279 nm. The molar concentration was calculated on the basis of $E_{1cm}^{1\%} = 6.67$.[13] The protein was diluted with concentrated buffer solution to give a final buffer with the following composition: 1.396 mM KH_2PO_4, 57.05 mM Na_2HPO_4, 70.32 mM NaCl, pH 7.4. This buffer was defined as the standard buffer, and was used in all experiments unless otherwise noted. The fluorescent probe, 1-anilinonaphthalene-8-sulfonate (ANS), was purchased as the sodium salt from Tokyo Kasei Co., Ltd.; N-phenyl-1-naphthylamine (NPN) was obtained from Wako Pure Chemical Industries, Ltd. Fatty acids were purchased from either Tokyo Kasei Co., Ltd. or Wako Pure Chemical Industries, Ltd. and were of the highest purity available (99%). 2,4,6-Trinitrobenzeneeulfonic acid (TNBS) and iodoacetamide were obtained from Wako Pure Chemical Industries, Ltd., and iodoacetamide was recrystallized from chloroform. 5,5-Dithiobis(2-nitrobenzoic acid) (DTNB) was obtained from Katayama Chemical Co., Ltd. Other chemicals of analytical grade were obtained from commercial sources, and were used without further purification.

Instrument——Fluorescent measurements were made with a Hitachi MPF-3 fluorescence spectrophotometer equipped with a recorder. All fluorescence emission spectra in this study are uncorrected. Ultraviolet absorption and circular dichroism (CD) spectral measurements were made with a Hitachi 124 spectrophotometer and a Jasco J-40A spectropolarimeter, respectively.

Fluorescence Titrations——The bindings of ANS and fatty acids to BSA were determined by the method described in the preceding paper.[11] BSA solution 2 ml (1.38×10^{-6} M) in pH 7.4 phosphate buffer was titrated by successive additions of 2 μl of ANS solution (1×10^{-3} M) in pH 7.4 phosphate buffer, using a microsyringe (MS-10, Jintan Terumo Co., Ltd.) at 27°. Binding of the fatty acids was determined by titrating a mixture of fatty acid and protein with ANS solution. In these experiments, the BSA concentration was 1.38×10^{-6} M in all cases. Because of their low solubility in pH 7.4 phosphate buffer, a concentrated solution of acid was prepared in methanol, and 0.1 ml was added slowly to 10 ml of BSA solution (1.38×10^{-5} M). After the addition of fatty acid, the BSA solution was diluted to 1.38×10^{-6} M with pH 7.4 phosphate buffer. The excitation and emission wavelengths for ANS were 375 and 465 nm, respectively. The fatty acid concentrations used were too low for micelles to form.[5b] To calculate the fractions of free and bound probe concentrations, fluorescence data at a high protein concentration, which provides excess protein binding sites, are required. In this study, the fluorescence titrations were carried out at several protein concentrations and the fluorescence at high protein concentration was obtained by an extrapolation method.[11] The probe-to-protein binding parameters were calculated using the Scatchard equation (1).[14]

$$\bar{v}/Df = nka - \bar{v}ka \qquad (1)$$

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where $\bar{\nu}$ is the number of moles of bound probe per mole of BSA, Df is the concentration of free probe, n is the number of binding sites on BSA, and ka is the association constant of the probe with BSA. The competitive binding of fatty acids to BSA was determined using equation (2).[11,15]

$$kb = \frac{ka(n-\bar{\nu}) - \bar{\nu}/Df}{Bt/Pt-n+\bar{\nu}+\bar{\nu}/Df(1/ka)} \times \frac{1}{(\bar{\nu}/Df)Pt} \tag{2}$$

where kb is the association constant for competitor, Pt is the total concentration of protein, and Bt is the total concentration of competitor.

CD Measurements——In order to obtain good CD spectra, the path length of the CD cell for the far-ultraviolet spectral region (200—250 nm) was 10 mm. The CD spectra were obtained by serially adding 2—10 μl of fatty acids in methanol to 2.0 ml of 1.38×10^{-6} M BSA solution in pH 7.4 phosphate buffer at room temperature (15—18°). Observed ellipticity θ_{obsd}, was converted to mean residue ellipticity $[\theta]$, by means of the following equation (3).

$$[\theta] = \frac{\theta_{obsd} \times (\text{mean residue weight})}{10 \times (\text{path length in cell}) \times (\text{mg/ml of protein})} \tag{3}$$

where $[\theta]$ is the mean residue ellipticity at 208 nm. For this purpose the CD spectra were expressed as mean residue ellipticity, using a mean residue weight of 114. The α-helix contents were calculated by the method of Greenfield and Fasman, using equation (4).[16]

$$\alpha\text{-helix} = \frac{-[\theta]-4000}{33000-4000} \tag{4}$$

Partition Experiment——The partition coeficient was obtained by equilibrating 5.0 ml of pH 7.4 phosphate buffer containing 5×10^{-5} M ANS with 5.0 ml of $CHCl_3$ containing 1—2×10^{-3} M fatty acid at room temperature (29—24°) for 4 hr with shaking. $CHCl_3$ and the buffer were saturated with each other before use. After shaking, the concentration of the probe in the water phase was determined by spectrophotometry.

Iodoacetamide Treatment of BSA (IA-BSA)——Iodoacetamide (2 mol per mol of albumin) was added to a 1% solution of BSA. The reaction was allowed to continue at 2° for a week and the pH was maintained at 7.4. The albumin solution was then dialyzed exhaustively against distilled water at 2°. The solution was freeze-dried and allowed to stand in a cold room (2°). The sulfhydryl content of the albumin solution was determined by spectrophotometric titration with DTNB;[17] no reactive sulfhydryl was detected.

TNBS Treatment of BSA (TNBS-BSA)——This reaction was performed according to the method of Andersson et al.[18] TNBS (1 mol per mol of albumin) was added to 1×10^{-4} M IA-BSA solution in 0.2 M carbonate buffer, pH 8.0. The solutions were allowed to stand at room temperature (22—27°) overnight.

Sulfhydryl Determinations——The SH content of serum albumin was determined by disulfide exchange with DTNB as described by Janatova et al.[17] A stock solution of 0.01 M DTNB in 0.037 M phosphate buffer, pH 8.0, was prepared. The reaction mixture was then prepared by mixing 0.8 ml of the stock DTNB solution and 0.2 ml of a 0.025 M EDTA solution, pH 8.10, with 3.0 ml of albumin solution, pH 8.10. The absorbance of the reaction mixture was read against a blank solution at 412 nm after the solutions had been allowed to stand for 45 minutes at room temperature. The concentration of albumin in the reaction mixture was in the range of 0.5—1%.

Results and Discussion

Binding of Fatty Acid to BSA in the Presence of ANS

The fluorescence spectra of ANS–BSA complex, excited at 300 nm, are shown in Figs. 1 and 2. When ANS is added to a BSA solution, BSA fluorescence (λ_{max} 340 nm), which is a pure tryptophan fluorescence,[11] is quenched, while a second peak appears in the 465 nm region, which represents ANS fluorescence. It is clear that BSA fluorescence decreases after the addition of 1×10^{-5} M capric acid, whereas on further increasing the capric acid concentration, a blue shift and enhancement appeared. Palmitic acid also produced both quenching and the blue shift and enhancement of BSA fluorescence, the extents of the changes being relatively larger than in the case of capric acid. Qualitatively similar results were obtained with lauric acid and stearic acid. Therefore, it has been suggested that fatty acid binding

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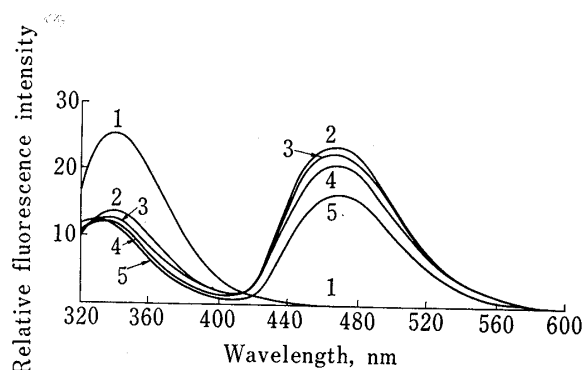
Fig. 1. Fluorescence Emission Spectra excited at 300 nm for Solutions containing 1.38×10^{-6} M BSA

1: in the absence of ligand.
2: in the presence of 2×10^{-6} M ANS.
3: in the presence of 2×10^{-6} M ANS and 1×10^{-5} M capric acid.
4: in the presence of 2×10^{-6} M ANS and 2×10^{-5} M capric acid.
5: in the presence of 2×10^{-6} M ANS and 5×10^{-5} M capric acid.
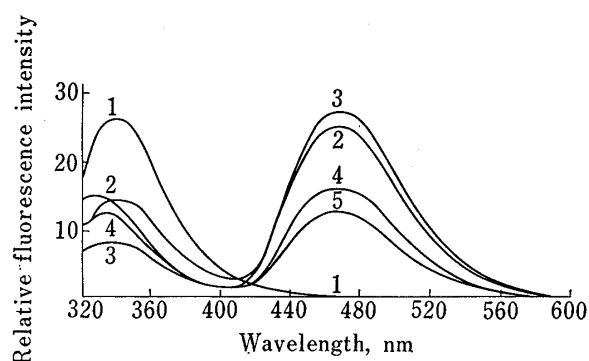
Fig. 2. Fluorescence Emission Spectra excited at 300 nm for Solutions containing 1.38×10^{-6} M BSA

1: in the absence of ligand.
2: in the presence of 2×10^{-6} M ANS.
3: in the presence of 2×10^{-6} M ANS and 5×10^{-6} M palmitic acid.
4: in the presence of 2×10^{-6} M ANS and 1×10^{-5} M palmitic acid.
5: in the presence of 2×10^{-6} M ANS and 2×10^{-5} M palmitic acid.

sites may be present in the immediate vicinity of at least one of the tryptophans of BSA,[19] and that the blue shift and enhancement are caused by the more hydrophobic environment for tryptophan in the fatty acid-BSA complexes than in the non-complexed BSA.[20] Thus, the tryptophan perturbations may be due to fatty acid-induced conformational changes in the probe binding sites. As the fatty acid concentration increases, the ANS fluorescence (λ_{max} 465 nm) decreases, but the wavelength of maximum emission does not change (Figs. 1, 2). However, in the case of palmitic acid, the fluorescence of ANS bound to BSA was enhanced at 5×10^{-6} M palmitic acid, 1.38×10^{-6} M BSA, and 1—2×10^{-6} M ANS. It has previously been suggested that this effect is due to an increase in available sites as a consequence of the combination of fatty acids with protein.[5b] Further, the partition coefficient of ANS was almost zero in the absence, as well as in the presence, of the fatty acids used in this experiment, and these results indicate that fatty acids are not capable of solubilizing ANS in a nonpolar environment.[1] Therefore, BSA may bind more ANS in the presence of palmitic acid.

A previous paper showed that ANS binds to BSA in a molar ratio of approximately $3 : 1$.[11] The binding parameters of capric acid, lauric acid, and palmitic acid to BSA were studied using ANS as a probe. The binding affinities of fatty acids are characterized by the association constant calculated from eq. (2). The method of titration used assumed that the quantum yield of ANS bound to BSA is the same in the presence or absence of fatty acids. (Orlov et al. have reported that fatty acids did not affect the fluorescence of either ANS or NPN bound to BSA.[21] The results for capric acid are shown in Fig. 3 and Table I. The experiments were carried out at four capric acid concentrations. The points show experimental data and the lines are theoretical plots generated by eq. (2). The theoretical curves shown in Fig. 3 are for $ka=1.1 \times 10^{6}$ M^{-1}, $Pt=1.38 \times 10^{-6}$ M, $n=2.9$ and using the association constants given in Table I for capric acid. The data are consistent with simple competition between capric acid and ANS for the same sites on the protein. Thus, the mechanism of inhibition of ANS–BSA complex formation by capric acid may

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TABLE I. Summary of the Association Constants[a]

Fatty acid	Bt[b]	This study $k \times 10^{-5}$ (M^{-1})		Literature n_1 n_2 $k_1 \times 10^{-5}$ $k_2 \times 10^{-5}$ Method[c]					
Capric acid	5×10^{-6}	2.7	6—7			0.6		ED, pH 6.8,	$2^{\circ[i]}$
	1×10^{-5}	2.0				1.03		ED, pH 7.4,	$37^{\circ[e]}$
	2×10^{-5}	1.2							
	5×10^{-5}	1.0							
Lauric acid	2×10^{-5}	3.6	6—7			2.3		ED, pH 6.8,	$2^{\circ[i]}$
			3	3		1.56	0.156	EP, pH 7.4,	$37^{\circ[f]}$
Palmitic acid	2×10^{-5}	6.9	3	3		288	5.98	EP, pH 7.4,	$23^{\circ[f]}$
			3	3		67.8	5.0	EP, pH 7.4,	$37^{\circ[f]}$
						340	81	EP, pH 7.4,	$37^{\circ[10]}$
			1.3			22		ED, pH 8.0,	$5^{\circ[18]}$

 a) k_1 and k_2=association constants; n_1 and n_2=numbers of binding sites.
 b) Total competitor in mole per liter.
 c) ED=equilibrium dialysis; EP=equilibrium partition.
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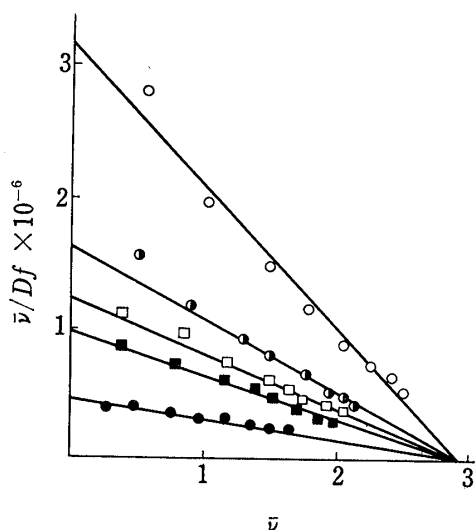 f) A.A. Spector, K. John and J.E. Fletcher, J. Lipid Res., 10, 56 (1969).
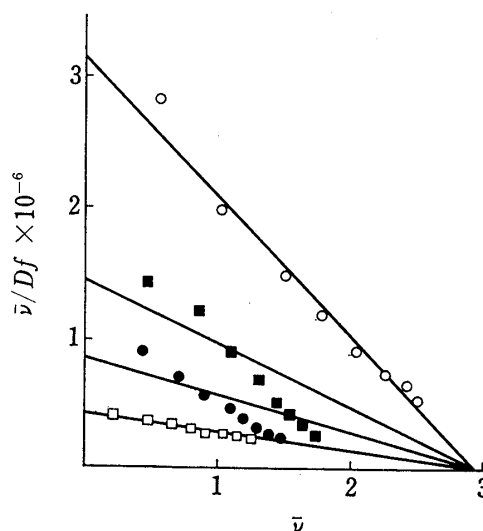

Fig. 3. Scatchard Plots for ANS and BSA binding at 27° and pH 7.4

—○— : in the absence of capric acid.
—◑— : in the presence of 5×10^{-6} M capric acid.
—□— : in the presence of 1×10^{-5} M capric acid.
—■— : in the presence of 2×10^{-5} M capric acid.
—●— : in the presence of 5×10^{-5} M capric acid.

Fig. 4. Scatchard Plots for ANS and BSA binding at 27° and pH 7.4

—○— : in the absence of lauric acid.
—■— : in the presence of 5×10^{-6} M lauric acid.
—●— : in the presence of 1×10^{-5} M lauric acid.
—□— : in the presence of 2×10^{-5} M lauric acid.

involve competition for the same binding sites. As the concentration of capric acid increases, the association constant for capric acid decreases from $2.7 \times 10^5\,\text{M}^{-1}$ to $1.0 \times 10^5\,\text{M}^{-1}$. This may be a reflection of the cumulative effects of the electrostatic factors, which have been neglected.[15] This also suggests that the polarity in the vicinity of the binding sites for ANS may be decreased in the presence of capric acid, judging from the blue shift of tryptophan fluorescence. Similar changes in ANS binding occurred when lauric acid or palmitic acid was added instead of capric acid (Figs. 4, 5). The theoretical curves shown in Fig. 4 are for $ka=1.1 \times 10^6\,\text{M}^{-1}$, kb of lauric acid $=3.6 \times 10^5\,\text{M}^{-1}$, $Pt=1.38 \times 10^{-6}\,\text{M}$ and $n=2.9$, and in Fig. 5 for $ka=1.1 \times 10^6\,\text{M}^{-1}$, kb of palmitic acid$=6.9 \times 10^5\,\text{M}^{-1}$ and $n=2.9$. In the case of $2 \times 10^{-5}\,\text{M}$ fatty acid, the experimental data are consistent with the theoretical

curves. However, in the case of 5×10^{-6} M and 1×10^{-5} M lauric acid or palmitic acid, the theoretical curves do not coincide with the experimental data. Eq. (2) is derived by assuming that two ligands are adsorbed at equivalent and independent sites, and the electrostatic interactions between successively bound anions are neglected. Thus, the question arises as to whether any electrostatic correction is neccessary in our case. It has been reported that there is good agreement between the experimental data and the theoretical curve without electrostatic correction.[11,22] Thus, it appears that the effect of electrostatic interaction is
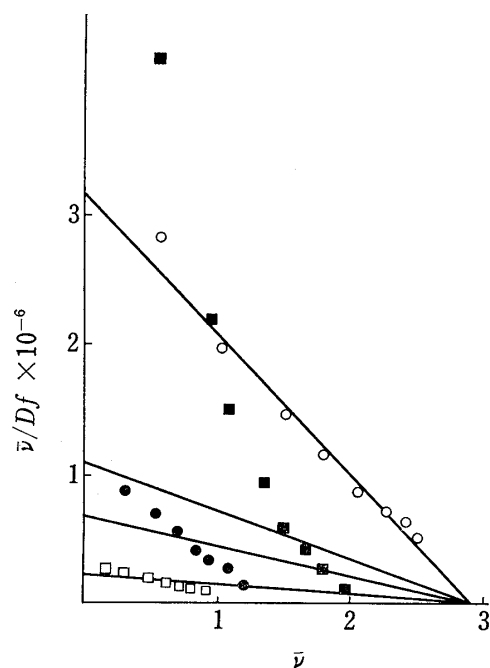
Fig. 5. Scatchard Plots for ANS and BSA binding at 27° and pH 7.4

—○— : in the absence of palmitic acid.
—■— : in the presence of 5×10^{-6} M palmitic acid.
—●— : in the presence of 1×10^{-5} M palmitic acid.
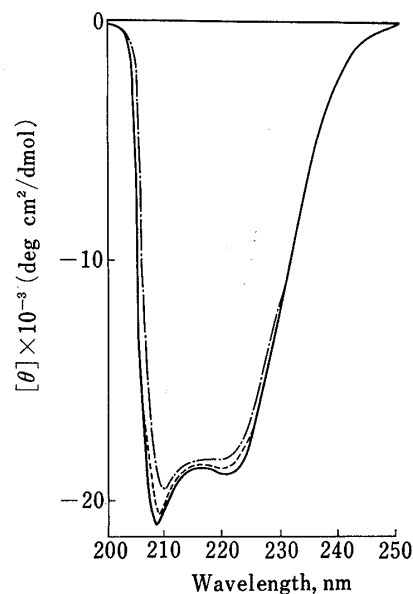—□— : in the presence of 2×10^{-5} M palmitic acid.

Fig. 6. CD Spectra of BSA in pH 7.4, Phosphate Buffer

———— : in the absence of palmitic acid.
- - - - - : in the presence of 1×10^{-5} M palmitic acid.
—·—·— : in the presence of 1.4×10^{-4} M palmitic acid.

TABLE II. Percentage of α-Helical Content of BSA[a] in the Presence of Fatty Acids

Fatty acid		α-Helix (%)
Control[b]	0	58
Capric acid	5 $\times 10^{-6}$ M	58
	1 $\times 10^{-5}$ M	58
	2 $\times 10^{-5}$ M	58
	5 $\times 10^{-5}$ M	57
	1.4 $\times 10^{-4}$ M	55
Lauric acid	5 $\times 10^{-6}$ M	57
	1 $\times 10^{-5}$ M	56
	2 $\times 10^{-5}$ M	56
	1.4 $\times 10^{-4}$ M	54
Palmitic acid	5 $\times 10^{-6}$ M	56
	1 $\times 10^{-5}$ M	56
	2 $\times 10^{-5}$ M	55
	1.4 $\times 10^{-4}$ M	51

a) All solutions contained 1.38×10^{-6} M BSA.
b) In the absence of fatty acid.

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small. Scatchard plots are derived by assuming that each of the multiple binding sites of a macromolecule competes independently for the available ligand. The theoretical basis of the model may be invalid in the case of binding of lauric acid or palmitic acid to BSA. The displacement of ANS by lauric acid or palmitic acid might result from either steric hindrance or a fatty acid-induced conformational change. Although the reason why the theoretical curves coincide with the experimental data only in the case of 2×10^{-5} M lauric acid or palmitic acid is not clear, palmitic acid at 5×10^{-6} or 1×10^{-5} M may bind to different sites from ANS, but may bind largely to the same sites at 2×10^{-5} M. A comparison of published data with the present data (Table I) shows that the values of the association constants obtained for capric acid and lauric acid seem to be good agreement with the primary association constant (k_1) given in the literature, but this is not so with palmitic acid. The association constant obtained for palmitic acid seems to be in good agreement with the secondary association constant (k_2) given in the literature. On the basis of the above discussion, the binding sites for ANS may be identical with the strong sites for capric acid or lauric acid, whereas ANS competes for the secondary sites of palmitic acid.

Far-Ultraviolet CD of the Fatty Acid-BSA System

The far-ultraviolet CD spectra of BSA (Fig. 6) illustrate the effect of palmitic acid concentration. The spectra were analyzed in terms of the secondary structure of the protein according to the procedure of Greenfield and Fasman.[16] Table II shows that palmitic acid and lauric acid produced a decrease in the apparent α-helical content of BSA at all concentrations tested. Capric acid had no effect at 5×10^{-6}—2×10^{-5} M, but it produced a decrease of α-helix when more than 5×10^{-5} M was present. These pilot observations indicate that the binding of fatty acids causes a marked change in the secondary structure of the protein, but demonstrate that capric acid had little effect on the secondary structure of BSA in the range of concentration used (5×10^{-6}—5×10^{-5} M). This also suggests that the difference between theory and experiment for palmitic acid and lauric acid in the binding experiments may be due to conformational change of BSA upon the binding of these fatty acids.

Displacement of the Probes from BSA by Fatty Acids

The fluorescence spectra of the NPN-BSA complexes, excited at 350 nm, are shown in Fig. 7. As the ANS concentration increased, the NPN fluorescence (λ_{max} 410 nm) decreased, and concurrently ANS fluorescence (λ_{max} 465 nm) increased. This indicates that the decrease in NPN flnorescence produced by ANS is due to displacemement of NPN from BSA. As proposed in a previous paper[11] the same regions on the protein molecule appear to be involved in the binding of both probes.

Figures 8 and 9 illustrate the effects of capric acid, lauric acid, palmitic acid and stearic acid on the fluorescence of ANS or NPN bound to BSA. Since the maximum numbers of binding sites (n) per BSA molecule for ANS and NPN are 2.9 and 2.0,[11] ANS and NPN were added in amounts of 3 mol/mol and 2 mol/mol albumin, respectively. Capric acid produced a moderate reduction in ANS fluorescence when more than 1 mol was present, whereas lauric acid produced a much greater reduction at all concentrations tested. Palmitic acid and stearic acid had no effect when only 1 mol was present, but produced a reduction in ANS fluorescence when larger amounts were added. Only when the molar ratio exceeded 4 did palmitic acid produce appreciable quenching. Though less lauric acid than palmitic acid or stearic acid is bound to albumin, lauric acid produced much greater reductions in ANS fluorescence than palmitic acid or stearic acid at molar ratios between 1 and 5. One explanation is that capric acid or lauric acid binds to the same sites as ANS or in the vicinity of the ANS sites, whereas the first few moles of palmitic acid or stearic acid bind to different sites. Similarly, Fig. 9 shows that the effects of these fatty acids on the fluorescence of NPN bound to BSA resemble those with ANS, but in this case palmitic acid produced a reduction even when 1 mol was present, possibly due to an allosteric change of BSA conformation.

In order to confirm that the first few moles of palmitic acid or stearic acid bind to different binding sites from ANS, the binding of fatty acid to IA–BSA and TNBS–BSA was studied. Long-chain fatty acids are known to protect the albumin sulfhydryl group against oxidation, and one of the primary fatty acid binding sites may be located near this sulfhydryl group.[9] The influence of fatty acids on the ANS fluorescence of solutions containing 3×10^{-6} M ANS and 1×10^{-6} M IA–BSA was studied at pH 7.4 and 27°. The fatty acids all
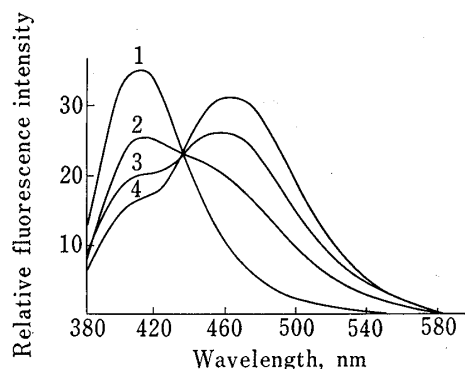
Fig. 7. Fluorescence Emission Spectra excited at 350 nm for Solutions containing 2×10^{-6} M NPN and 1×10^{-6} M BSA

1: in the absence of ANS.
2: in the presence of 1×10^{-6} M ANS.
3: in the presence of 2×10^{-6} M ANS.
4: in the presence of 3×10^{-6} M ANS.

Fig. 8. Effects of Fatty Acids of Various Chain Lengths on the Fluorescence of ANS-BSA Complex (3×10^{-6} M ANS and 1×10^{-6} M BSA) at 27° in pH 7.4 Phosphate Buffer

The excitation and emission wavelengths used were 375 nm and 465 nm.
—○— : capric acid,   —●— : lauric acid,
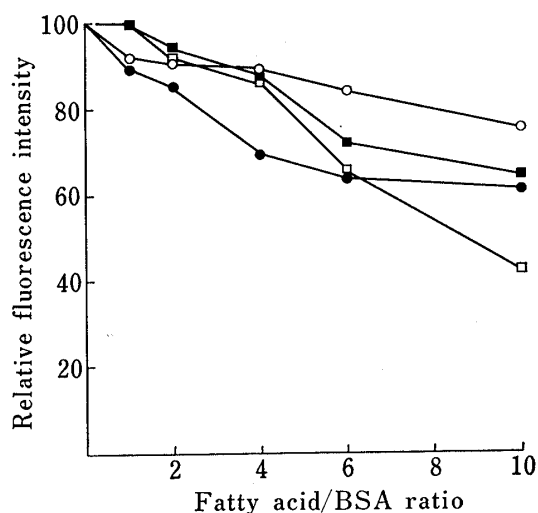—□— : palmitic acid,   —■— : stearic acid.
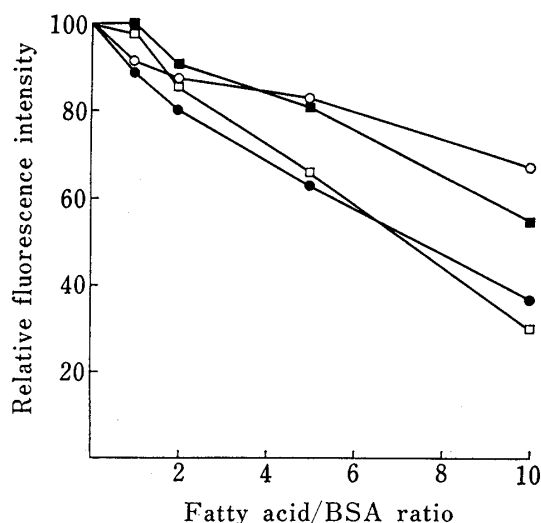
Fig. 9. Effects of Fatty Acids of Various Chain Lengths on the Fluorescence of NPN-BSA Complex (2×10^{-6} M NPN and 1×10^{-6} M BSA) at 27° in pH 7.4 Phosphate Buffer

The excitation and emission wavelengths used were 350 nm and 410 nm.
—○— : capric acid,   —●— : lauric acid,
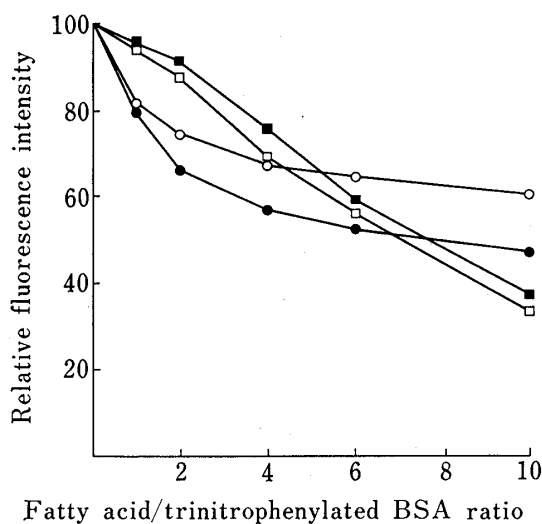—□— : palmitic acid,   —■— : stearic acid.

Fig. 10. Effects of Fatty Acids of Various Chain Lengths on the Fluorescence of ANS-TNBS-BSA Complex (3×10^{-6} M ANS and 1×10^{-6} M TNBS-BSA) at 27° in pH 7.4 Phosphate Buffer

The excitation and emission wavelengths used were 375 nm and 465 nm.
—○— : capric acid,   —●— : lauric acid,
—□— : palmitic acid,   —■— : stearic acid.

gave curves almost identical to those in Fig. 8. It was found that binding sites for fatty acids seemed to be unaffected, suggesting that one of the main fatty acid binding sites is not located near the single sulfhydryl group. Fig. 10 shows the influence of fatty acids on the ANS fluorescence of solutions containing TNBS–BSA. Palmitic acid or stearic acid produced a reduction of the ANS fluorescence when 1 mol was present. Further, the binding parameters of ANS with TNBS–BSA are about the same as with native BSA, as determined by the fluorescence method. In the case of binding of ANS to TNBS–BSA, titrations were monitored by fluorometry with excitation at 305 nm, a wavelength at which TNBS–BSA and ANS show no appreciable absorption. These results indicate that one of the strongest binding sites for long-chain fatty acids may be blocked by TNBS. Capric acid or lauric acid also produced a much greater quenching of ANS fluorescence than in native BSA. These results suggest that capric acid or lauric acid may be weakly bound to the TNBS binding sites of BSA, and this affinity labeling may result in an increase in competitive binding between ANS and these fatty acids.

King discovered that under special conditions pepsin would specifically cleave a single bond in BSA near the middle of the molecule to give the fragments P–B (1—306) and P–A (307—585).[23] Reed et al. showed that both fragments have high helical contents (63 and 57%) as determined by CD, indicating that they retain substantial secondary structure.[10] They reported that the fragment P–A has the strong binding sites for palmitate, with an association constant about three-fifths of that of BSA, while the fragment P–B shows weaker affinity, less than a quarter of that of BSA. These experiments demonstrate that the strong binding sites for long-chain fatty acid are located in the carboxyl-terminal half of the molecule. TNBS reacts preferentially and quantitatively with ε-amino groups of albumin under mild conditions to give the corresponding trinitrophenyl derivatives.[24] Andersson et al. showed that one of most reactive sites with TNBS is involved in the binding of palmitate with BSA, and that this site contains lysine which can be labeled with TNBS.[18] These results provide strong evidence that one of the strongest binding sites for long-chain fatty acids is the TNBS site, located in the carboxyl-terminal half of the molecule, which does not contain tryptophan residues.

Based on the above discussion, it seems likely that the mechanism of inhibition of the probe-BSA binding by capric acid may involve competition for the same binding sites, and, in contrast, such decreases in probe binding with BSA in the presence of lauric acid, palmitic acid or staric acid are not due simply to displacement of the probes by fatty acids from the probe-binding sites, but could also involve a conformational change of the probe-binding sites caused by the interaction of probes and fatty acids.

23) T.P. King, Arch. Biochem. Biophys., **156**, 509 (1973).
24) A.R. Goldfarb, Biochemistry, **5**, 2574 (1966).