Binding and proximity relationships of hydrophobic ligands on human serum albumin have been studied using absorption, fluorescence, circular dichroism, and electron paramagnetic resonance spectroscopy. The ligands studied were bilirubin, two conjugated linear polyene fatty acids, cis-parinaric acid and cis-eleostearic acid, and three nitroxide derivatives of stearic acid with doxyl groups at positions 5, 10, and 12, respectively. Binding of polyene fatty acids was monitored by absorption peak shifts, induced circular dichroism, enhancement of fluorescence, and energy transfer between albumin's single tryptophanyl residue and the polyene chromophore. Induced dichroism studies indicate excitonic ligand-ligand interaction between bound fatty acids. Fluorescence enhancement of cis-parinaric acid was analyzed using a stepwise multiple equilibrium model, and six binding constants in the range $10^8$ to $10^6$ were obtained, in agreement with previous measurements for other fatty acids. The temperature dependence of the equilibrium constants indicates that the binding enthalpy is nearly zero. Fluorescence energy transfer was similarly used to quantitate bilirubin binding to albumin. Energy transfer, nitrooxide quenching of fluorescence, and electron paramagnetic resonance spectroscopy were used to elucidate binding geometries which support and extend proposed structural models for albumin. It is suggested that the first two fatty acids bind side-by-side in an antiparallel fashion in domain III of human serum albumin.

Albumin is the most plentiful protein in mammalian plasma. A monomeric protein of molecular weight 68,000, albumin is the physiological carrier of fatty acids, lyssolecithin, and bilirubin, and it also carries many hydrophobic drugs. Several mammalian albumins have recently been sequenced (Behrens et al., 1975; Brown et al., 1971). Based on sequence data, analysis of peptide fragments, and hydrodynamic measurements, a partial domain model for the tertiary structure of the protein has recently been proposed (Anderson and Weber, 1969; Pederson and Foster, 1969; Brown, 1976). According to this model, albumin is comprised of three major domains (see Fig. 15) which are in turn comprised of subdomains. The subdomains consist of cylindrical segments formed by apposition of a helical regions. These helical regions form elongated pockets for fatty acids with hydrophobic residues lining the inner surfaces and several positively charged groups at the opening, near the expected location of the fatty acid carboxyl groups. The strongest binding site for long chain fatty acids is thought to be in domain III. The single tryptophan residue in human serum albumin lies in domain II.

Several investigators (Goodman, 1958; Spector et al., 1968; Fletcher et al., 1970; Arvidsson et al., 1971; Spector et al., 1971; Ashbrook et al., 1975) have measured the binding of long chain fatty acids to albumins by using partitioning of fatty acids between an organic phase such as heptane and an aqueous medium containing the protein. This method of measurement was used in part because long chain fatty acids dialyze very poorly. Specific assumptions must be made concerning the partitioning of fatty acids between organic solvents and water and the activity coefficients of fatty acids in water, and these are subjects of much current debate (Mukerjee, 1965; Tanford, 1973; Smith and Tanford, 1973). Organic solvents such as heptane bind to albumin, and this might complicate the analysis. In any event, corroboration by a single phase method would be helpful.

Sklar et al. have recently introduced conjugated linear polyene fatty acids as probes of membrane structure and of lipid-protein interactions. The important features of these probes have been described in detail elsewhere (Sklar et al., 1975, 1977, a and b), and their binding to bovine serum albumin has been studied (Sklar et al., 1977c). Briefly, these probes are isomers of 9,11,13,15-octadecatetraenoic acid (parinaric acid) and 9,11,13-octadecatrienoic acid (eleostearic acid). Parinaric acid fluoresces when bound to albumin, but not when it is in water, and the absorption spectra shift to longer wavelengths. The enhancement of fluorescence was used by Sklar et al. to quantitate binding, but binding constants were obtained only for the 3rd to 5th mol bound. Binding of PnA to bovine serum albumin results in an increase.

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duced chroomophile circular dichroism. When 2 mol of PnA are bound per mol of protein ($\bar{v} = 2$), a negative component of the circular dichroism is observed, which was attributed to excitonic ligand-ligand interaction (Sklar et al., 1977c). The emission spectrum of tryptophan overlaps the absorption spectra of cPnA and cEsA, and this feature allowed measurement of distances from the two tryptophans to the first fatty acid binding site by application of the Förster-Dexter theory of energy transfer.

In this study we extend these absorption, circular dichroism, and fluorescence measurements to human serum albumin. To supplement this information, we also employ electron paramagnetic resonance spectroscopy of nitroxy fatty acids bound to human serum albumin and quenching of the fluorescence of cPnA by nitroxy fatty acids. These measurements permit determination of binding affinities under various conditions, and permit conclusions regarding proximity of ligands to each other and to the single tryptophanyl residue of human serum albumin.

Albumin carries bilirubin, the breakdown product of heme, to the liver for conjugation with glucuronic acid and subsequent excretion. Albumin has one very strong binding constant ($10^6 \text{M}^{-1}$) and two weaker binding constants for bilirubin ($10^5 \text{M}^{-1}$). It has been observed clinically and in experimental systems that when the bilirubin to albumin ratio exceeds 1, bilirubin binds to cell membranes. This is a common occurrence in newborn infants, particularly if premature, and the deposition of bilirubin in the basal ganglia of the brain leads to a clinical syndrome, known as kernicterus, involving neurological impairment. There is therefore great clinical interest in understanding the binding of bilirubin to albumin and the effects of drugs and other competitors on this binding.

We employ energy transfer between tryptophan and bilirubin to extend the measurements of Chen (1973) on binding of bilirubin and use energy transfer between cPnA and bilirubin to further specify distance relationships between bound ligands.

**Materials and Methods**

See Miniprint.²

**Results and Discussion**

Absorption Spectroscopy—Fig. 1 shows absorption spectra of cPnA ($2 \times 10^{-5} \text{M}$) in 0.02 M sodium phosphate, pH 7.4, as aliquots of human serum albumin are added. As shown in Fig. 1A, cPnA in buffer has its first absorption maximum, P1, at about 321.2 nm, and the value for the ratio of the absorbance at the first valley, V1, to the value of the absorbance at the second peak, P2, is about 0.52. As aliquots of human serum albumin are added, the observed spectrum reflects the superposition of spectra due to bound and free fatty acid. When about 0.12 mol of human serum albumin is added per mol of cPnA, the valley to peak ratio is greatest, indicating roughly equal amounts of bound and free fatty acid. This is consistent with the binding of between 4 and 5 mol of fatty acid/mol of human serum albumin. As more human serum albumin is added, P1 shifts to longer wavelengths and V/P drops again to a final value of about 0.52, indicating that nearly all of the PnA is bound under these conditions. The positions of peak P1 and the values of V1/P2 are plotted in Fig. 2, B and A, respectively. The absorption maximum of cEsA exhibits a similar shift upon binding, as shown in Fig. 2C. The peaks of the cEsA absorption spectrum are not sufficiently resolved to permit measurement of valley to peak ratios. These results are very similar to those previously reported for bovine serum albumin (Sklar et al., 1977c).

Circular Dichroism Spectra of cPnA Bound to Human Serum Albumin—cPnA is not optically active. An intense CD is observed in the region of cPnA absorption for several cPnA-protein complexes (Sklar et al., 1975, 1977c). Fig. 3 shows this induced CD for the cPnA-HSA complex with $\bar{v} = 1$ and $\bar{v} = 2$. The CD of an equal molar HSA-oleic acid solution has been subtracted from the CD of the complex. The absorption spectrum for $\bar{v} = 2$ and the ratio $\Delta \epsilon / \epsilon$ are also shown for comparison. The absorption spectrum for $\bar{v} = 1$ is very similar to that for $\bar{v} = 2$.

Several features of these CD spectra are noteworthy. When $\bar{v} \leq 1$, the CD is entirely positive and has the same general features as the absorption spectrum. The magnitude of this positive CD is very similar to that observed for the cPnA-BSA complex (Sklar et al., 1977c). A comparison of the absorption and CD spectra for the cPnA-HSA complex shows that the CD is blue-shifted relative to the absorption and that the positive intensities of the vibronic components are appreciably altered. In the CD the higher energy vibronic components have relatively more intensity. When $\bar{v} > 1$, there is a negative component in the CD spectrum at long wavelengths and an increase in the positive component at short wavelengths. This behavior is qualitatively similar to that observed for the

² Portions of this paper (including "Materials and Methods," and Figs. 1, 2, 5, 7, 9, 11, 13, and 14) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, Md. 20014. Request Document No. 78M-9, cite author(s), and include a check or money order for $2.25 per set of photocopies.
BSA-cPnA complex, but the magnitude of the negative component of the CD is smaller in the case of human serum albumin by a factor of about 3 (Sklar et al., 1977c).

The induced CD observed for the $\tilde{v} = 1$ complex is due to the adoption of a chiral geometry by the fatty acid tetraene chromophore. The strong electric dipole transition near 310 nm has no magnetic transition moment in the absence of the chiral perturbation. This perturbation may be viewed as a mixing of low energy magnetic dipole allowed states with the electric dipole allowed state and vice versa. The resulting mixed states exhibit circular dichroic transitions. One of the magnetic dipole transitions which will be mixed with the 310 nm transition is the ground state to excited $\tilde{A}_g$ transition whose vibrationless origin is at about 350 nm (Hudson and Kohler, 1974; Sklar et al., 1977a; Andrews and Hudson, 1978). The vibronic structure of this forbidden transition extends to about 290 nm. This state will give rise to a band centered at about 330 nm with a rotatory strength opposite in sign to that of the 310 nm band. This results in the shift of the low energy vibronic bands at 308 and 325 nm and a relative decrease in their intensity compared to the higher energy bands.

The CD spectrum for the complex with two bound PnA's has a pattern characteristic of exciton coupling between the tetraene chromophores, i.e., a negative long wavelength component and a positive short wavelength component superimposed on the positive CD due to the chiral chromophore geometry. The CD spectrum for the human serum albumin complex with one cPnA molecule ($\tilde{v} = 1$) is unaffected by the addition of oleic acid. This is also the case for the BSA-PnA complex (Sklar et al., 1977c). This demonstrates that the new features of the $\tilde{v} = 2$ spectrum are associated with the presence of two bound chromophores rather than being a property of the chromophore in the second binding site. The exciton splitting estimated from the $\tilde{v} = 2$ spectrum is 3500 to 5500 cm$^{-1}$. If the two transition dipoles are oriented so that the exciton splitting is maximized for a given relative distance, then that distance is 5 to 7 Å; for any other transition dipole orientation the chromophores must be closer together. This is direct evidence that the first two fatty acid binding sites are very close together.

It should be noted that this exciton splitting is due to the transition dipole-transition dipole interaction of the strong transition ($\tilde{f} \sim 1.3$) to the second excited singlet state. The low energy $\tilde{A}_g$ state has a transition dipole which is about 50 times smaller ($\tilde{f} \sim 0.025$) and will therefore have an exciton interaction which is about 2500 times smaller. The fluorescence originates in this $\tilde{A}_g$ excited state (Sklar et al., 1977a) and therefore the fluorescence of the bound PnA will not be affected by this exciton interaction.

**Fluorescence Enhancement of Bound PnA**—The fluorescence enhancement associated with the binding of cPnA to human serum albumin was used to measure binding constants under a variety of conditions. Fig. 4 shows the enhancement of cPnA fluorescence as PnA is added to a constant amount of human serum albumin. We note that in this concentration range (1 x 10$^{-5}$ M), the 1st mol of cPnA is essentially all bound, and the amount of fluorescence is directly proportional to the amount bound. Fluorescence intensity increases nearly linearly until 5 or 6 mol of cPnA are added, and then it levels off abruptly. We therefore assume that for any amount of cPnA bound, the fluorescence intensity is directly proportional to the amount bound. cPnA was added to human serum albumin at mole ratios ranging from 0.1 to 10, and at human serum albumin concentrations ranging from 1.0 x 10$^{-5}$ M to 1.6 x 10$^{-7}$ M. Knowing the amount of fluorescence present for a certain concentration of bound cPnA, the observed intensities at various concentrations and mole ratios were used to determine the amount of bound and free fatty acid in each case. Fig. 5 and Table I summarize the results of these binding studies and their interpretation in terms of Equation 7.

![Fig. 4. Enhancement of PnA fluorescence on binding to human serum albumin and quenching of human serum albumin fluorescence by polyene fatty acids.](http://www.jbc.org/)
The binding is apparently only weakly temperature-dependent. This implies that the enthalpy of binding is very small, and the free energy of binding is due primarily to an entropy increase upon binding, which is a general characteristic of so-called "hydrophobic" interactions in other systems (Klotz and Urquhart, 1949; Tanford, 1973). Similar experiments have shown very little change in the binding affinity as the salt concentration is varied over a range from 0 to 300 mM. Removal of Mg$^{2+}$ results in a slight increase in binding affinity. The binding constants obtained are quite similar to those obtained by Ashbrook et al. (1975), despite differences in experimental design and use of a different fatty acid. This lends corroboration to their findings.

The binding constants given in Table I refer to the binding affinity for the $i$th cPnA molecule to human serum albumin with $i - 1$ cPnA molecules bound. There is no direct connection between these binding constants and the affinities for individual sites unless sequential binding is assumed. An alternative description in terms of site affinities can be obtained from Equation 1 which is valid in the absence of site-site interactions. The fit obtained with Equations 7 and 8 (which involve the same number of parameters) is equally good. The RMS error is the root mean square deviation between the measured values of $\bar{r}$ and those calculated with Equation 7 and the binding constants given. The enthalpy change on binding to the first site is calculated from the increase upon binding, which is a general characteristic of so-called "hydrophobic" interactions in other systems (Klotz and Urquhart, 1949; Tanford, 1973). Similar experiments have shown very little change in the binding affinity as the salt concentration is varied over a range from 0 to 300 mM. Removal of Mg$^{2+}$ results in a slight increase in binding affinity. The binding constants obtained are quite similar to those obtained by Ashbrook et al. (1975), despite differences in experimental design and use of a different fatty acid. This lends corroboration to their findings.

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occur for a very small range of the relative orientation variables and which requires that there is no relative orientational motion of the chromophores during the time between excitation and emission. This is because the distance measurements only depend on the sixth root of $\kappa^2$. For instance, if $\kappa^2$ is 4 instead of $\frac{1}{2}$, the distance will be underestimated by 35%. Similarly, if $\kappa^2$ is 0.1, the distance will be overestimated by 35%. To eliminate the possibility of large overestimates of distances, which occur when $\kappa^2$ is close to 0, we employed quenching of human serum albumin fluorescence by the nitroxide fatty acids 5 doxyl stearate (5 NS), 10 doxyl stearate (10 NS), and 12 doxyl stearate (12 NS). (In our notation, the number indicates the carbon bearing the doxyl group.) Our data is in general agreement with the data of Morrisett et al. (1975) on bovine serum albumin in two respects: 1) the quenching is greatest for 5 NS and 2) quenching increases with $\tilde{r}$ more rapidly when $\tilde{r} = 3$ or 4 than when $\tilde{r} < 3$. The 12 NS fatty acid is most nearly analogous to cPnA due to the position of its substituent. At all mole ratios, the quenching by 12 NS is much less than that of eleostearic acid. The quenching by nitroxide fatty acids is isotropic and very efficient for distances less than about 8 Å but is inefficient for large distances. The low quenching of tryptophan emission observed for 12 NS therefore indicates that the first two fatty acids are not very close to the tryptophan and that $\kappa^2$ does not have a vanishingly small value for these ligands.

Our analysis of the cPnA and cEsA tryptophan quenching curves in terms of ligand to tryptophan distances is based on the assumption of sequential binding, i.e. discrete binding sites with associated binding constants which are well separated. The cEsA quenching curve of Fig. 4 shows that the second and third ligands bind sequentially with respect to the first and second ligands. Alternative descriptions of the binding in which the intrinsic site binding constants are, in fact, averages over several sites. This does not affect our basic conclusion, however, which is that the third and fourth fatty acid ligands bind closer to the tryptophan than the first and second ligands. If the binding is nonsequential, then the first two sites must be even further from the tryptophan and the third and fourth sites must be even closer. The conclusion that the first two fatty acids bind about equally distant from the tryptophan is supported by independent experiments which show that these two fatty acids are close together in the complex.

The energy transfer data presented here may be compared with the data of Sklar et al. (1977c) in their studies of bovine serum albumin. Binding of a single oleic acid to bovine serum albumin reduces the protein fluorescence. This effect is not seen in human serum albumin until $\tilde{r}$ is greater than 3. When $\tilde{r} = 1$ for cEsA, there is virtually no additional quenching of bovine serum albumin, while there is sizable quenching of human serum albumin. This difference may be ascribed in part to the fact that the emission spectrum of human serum albumin is shifted to shorter wavelengths than the bovine serum albumin emission spectrum, and spectral overlap with cEsA is greater. Another reason for the relatively smaller degree of quenching of bovine serum albumin is that roughly half the emission intensity for bovine serum albumin is thought to be due to the tryptophanyl residue, not present in human serum albumin, which resides in domain I. It is to be expected that this tryptophanyl residue is very far from the first fatty acid bound, and would not be quenched effectively. In both human serum albumin and bovine serum albumin, the third and fourth fatty acids quench tryptophan emission better than the first two fatty acids. Both of these findings are consistent with the assignment of the two strongest binding sites to domain III in Brown's model (Brown, 1976) and subsequent binding to domain II.

**Quenching of the Emission of Bound cPnA by Nitroxide Fatty Acids**—The occurrence of a negative peak in the induced CD spectrum of cPnA bound to bovine serum albumin and human serum albumin has been interpreted in terms of ligand-ligand exciton interaction between bound fatty acids (Sklar et al., 1977c). In order to further probe geometric relationships between bound fatty acids, we have examined changes in the fluorescence and absorption spectra of cPnA bound to human serum albumin ($\tilde{r} = 1$) upon addition of nitroxide-labeled fatty acids. As shown in Fig. 8, 5 NS produces sizable quenching of fluorescence when $\tilde{r} = 5$ NS, whereas 10 NS, 12 NS, and oleic acid produce little or no quenching at this level of binding. The possibility that the decreased fluorescence of bound cPnA due to addition of 1 mol of 5 NS is due to displacement of the cPnA was excluded by observing that the cPnA absorption spectrum does not shift to shorter wavelength until at least 5 mol of 5 NS have

![FIG. 8. Quenching by nitroxide fatty acids of the fluorescence of cPnA bound to human serum albumin. A solution of human serum albumin ($1 \times 10^{-8}$ M) was prepared and cPnA was added in equimolar ratio. Excitation of cPnA fluorescence was performed at 330 nm (3 nm slit) and emission was collected at 420 nm (5 nm slit). Nitroxide fatty acids in ethanol were added, and changes in fluorescence intensity were recorded. As a control, a similar titration was performed with oleic acid.](http://www.jbc.org/)

**Table II**

**Distances between the tryptophanyl residue of human serum albumin and polyene fatty acid chromophores as determined by energy transfer**

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**Fig. 8.** Quenching by nitroxide fatty acids of the fluorescence of cPnA bound to human serum albumin. A solution of human serum albumin ($1 \times 10^{-8}$ M) was prepared and cPnA was added in equimolar ratio. Excitation of cPnA fluorescence was performed at 330 nm (3 nm slit) and emission was collected at 420 nm (5 nm slit). Nitroxide fatty acids in ethanol were added, and changes in fluorescence intensity were recorded. As a control, a similar titration was performed with oleic acid.
been added. This kind of experiment can be used to estimate the relative binding affinities of these ligands for human serum albumin. Absorption and fluorescence data in cases in which \(\tilde{r}\) ranges from 6 to 9 for oleic acid or nitroxide fatty acids suggest the following order of binding affinities: oleic acid \(\approx\) cPnA > 5 NS \(\approx\) 10 NS \(\approx\) 12 NS.

The polyene chromophore of cPnA is located toward the methyl end of the chain, and the best quenching occurs when the doxyl moiety is nearer to the COOH terminus. This suggests that for the first two fatty acids bound, the arrangement is roughly antiparallel side by side as shown in Fig. 15. The general features of these spectra are similar to those observed by Morrisett et al. (1975) in their study of bovine serum albumin. The spectral lines are broadened, reflecting the relative immobilization of the nitroxide moieties upon binding to the protein. Superimposed on these broad lines are much smaller sharp components due to the presence of unbound probe. We note that more fatty acids remains unbound in our experiment than in that of Morrisett et al. (1975) because we work at a protein concentration which is approximately an order of magnitude lower (5 \(\times\) 10\(^{-5}\) M). It is apparent from these spectra that 12 NS, which has its spin label near the methyl terminus, binds less strongly than 10 NS or 5 NS.

The model for human serum albumin proposed by Brown (1976) and the fluorescence data presented above suggest that two fatty acids may bind to a single domain. The fluorescence energy transfer measurements indicate that the first two bound fatty acids are equally distant from the tryptophan. The CD measurements and the efficient quenching of bound cPnA by bound 5 NS indicates that these two species bind close together. The positional dependence of this quenching indicates an antiparallel side by side arrangement. In an attempt to further examine this hypothesis, we recorded EPR spectra of pairs of different nitroxide fatty acids bound to human serum albumin. In each case, 1 mol of each type was added per mol of human serum albumin. The spectra of these combinations are shown in Fig. 9B. Close approach of two nitroxide moieties (about 10 Å) would be expected to produce spin-spin broadening of the spectra; no obvious broadening was observed. Evidence for ligand-ligand interaction can nevertheless be obtained by examination of sharp components in the spectra, which are due to unbound probe. As shown in Fig. 9B and Table III, the amount of unbound probe and hence the binding affinity when \(\tilde{r}\) = 2 depends on the particular pair of nitroxide fatty acids used, and not merely on their individual affinities when \(\tilde{r}\) = 1. For example, there is very little unbound probe when \(\tilde{r}\) = 1 for either 5 NS or 10 NS (<0.2% unbound 5 NS and 0.8% unbound 10 NS, respectively), while there is relatively weaker binding of 12 NS (4.0% unbound 12 NS). When \(\tilde{r}\) equals 2 for 5 NS, the percentage of unbound probe increases to 0.3%, as would be expected for sequentially decreasing binding constants for successive sites. Similarly, when \(\tilde{r}\) = 2 for 12 NS, then the amount of unbound probe increases to 0.4%. The surprising finding is that when \(\tilde{r}\) = 2 for 10 NS the amount of unbound probe is 12.9%, greater than that observed for 12 NS, despite the fact that 10 NS is much more strongly bound than 12 NS when \(\tilde{r}\) = 1. Moreover, the amount of unbound 10 NS when \(\tilde{r}\) = 2 is greater than the amount of unbound probe when the pair 10 NS + 12 NS is studied. We conclude that the binding affinity of a particular nitroxide fatty acid depends on the position in the chain of the nitroxide group on the other fatty acid bound. An interpretation for this finding can be given which is consistent with the geometrical information provided by the previously mentioned studies of nitroxide quenching of cPnA. The fatty acid binding sites on human serum albumin appear to have reasonably strict steric requirements. While there is only a slight dependence of the binding affinity on the position of a single nitroxide moiety into a binding site, it appears that there is not enough room for two nitroxide groups to approach each other closely without a substantial reduction in binding affinity. The extent of increase in the amount of unbound fatty acid for a given pair of nitroxide fatty acids with nitroxide groups at the \(j\)th and \(k\)th positions in their respective chains is probably related to the proximity of the \(j\)th carbon on a first chain to the \(k\)th carbon in a second chain. As depicted in Fig. 15, the proposed antiparallel side by side binding arrangement will result in relatively large steric hindrance for the pair 10 NS + 10 NS, and relatively less for the pair 5 NS + 5 NS, as is observed.

**Fig. 10.** Spectral overlap of bilirubin absorption with human serum albumin (HSA) and cPnA fluorescence. The dashed and dotted curves are the emission spectra of cPnA and human serum albumin, respectively. The solid curve is the absorption spectrum of bilirubin when bound to human serum albumin. The shaded areas represent regions of spectral overlap. It is apparent that cPnA and bilirubin have extremely strong spectral overlap, and that human serum albumin (i.e. tryptophan) and bilirubin have moderately strong spectral overlap.

**Table III: Binding of combinations of nitroxide fatty acids to human serum albumin**

<table>
<thead>
<tr>
<th>Nitroxide fatty acid</th>
<th>Unbound nitroxide fatty acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 NS</td>
<td>&lt;0.2%</td>
</tr>
<tr>
<td>10 NS</td>
<td>0.8%</td>
</tr>
<tr>
<td>12 NS</td>
<td>4.0%</td>
</tr>
<tr>
<td>10 NS + 5 NS</td>
<td>2.9%</td>
</tr>
<tr>
<td>10 NS + 12 NS</td>
<td>7.2%</td>
</tr>
<tr>
<td>10 NS + 10 NS</td>
<td>12.9%</td>
</tr>
<tr>
<td>5 NS + 12 NS</td>
<td>6.0%</td>
</tr>
<tr>
<td>12 NS + 12 NS</td>
<td>9.4%</td>
</tr>
<tr>
<td>5 NS + 5 NS</td>
<td>0.3%</td>
</tr>
</tbody>
</table>

Each fatty acid listed is present in an equimolar ratio to human serum albumin at 5 \(\times\) 10\(^{-4}\) M.
rubin are shown in Fig. 11, and relative emission intensities at 346 nm are plotted in Fig. 12. This quenching profile is quite similar to that observed by Chen (1973). It can be seen that bilirubin quenching is quite efficient (T = 66% when r = 1). From the data shown in Fig. 10, the value of the spectral overlap integral J is 1.74 X 10⁻¹⁴ and R₀° is 31 Å. Using the measured transfer efficiencies when r = 1 or 2, we calculate the apparent distances from the tryptophanyl residue to the first and second bilirubin chromophores to be 27 Å and 24 Å, respectively (see Table IV). As noted by Chen (1973), fluorescence quenching at different protein concentrations can be used to obtain binding constants. We have repeated his measurements and studied binding at 5°C, 23°C, and 37°C. Binding data were analyzed by a stepwise multiple equilibrium model and are shown in Fig. 13 and Table V. Results at 23°C compare well with those obtained by Chen (1973) and Levine (1977), and by other methods (Jacobsen, 1969) using a Scatchard model. Results over this range of temperatures suggest moderate temperature dependence of binding.

**Energy Transfer between cPnA and Bilirubin**—The absorption spectrum of bilirubin also overlaps the emission spectrum of cPnA as shown in Fig. 10. In this case the spectral overlap is extremely large, and the spectral overlap integral is 1.031 X 10⁻¹³. The value of R₀° is calculated to be 41 Å. Fig. 14 shows the quenching of the fluorescence of cPnA bound to human serum albumin (r = 1) as aliquots of bilirubin are added. A similar quenching profile is observed when 2 mol of cPnA chromophores and the first two bound bilirubin chromophores are added. As noted under “Materials and Methods,” the quantum yields of the first 2 bound cPnA mol are identical, and this allows calculation of distances between both bound cPnA chromophores and the first two bound bilirubin chromophores. As shown in Table IV, in each case, the fatty acid-bilirubin distance is large, ranging between 35 and 40 Å. This finding is consistent with the observation (Woolley and Hunter, 1970; Berde et al.) that the first two fatty acids bound to human serum albumin do not cause measurable displacement of bilirubin.

---

**Table IV**

**Distances from Bound Bilirubin to Polyene Fatty Acids and the Tryptophanyl Residue of Human Serum Albumin**

Apparent distances between chromophores are calculated using the transfer efficiencies derived from Figs. 12 to 14, similar curves for the case in which r = 2 for PnA (data not shown), and Equations 1 to 4. Values of the pertinent spectroscopic parameters are given in the text, and x² is set equal to % in each case.

<table>
<thead>
<tr>
<th>Donor-acceptor</th>
<th>R²[Å]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tryptophan-bilirubin</td>
<td>27</td>
</tr>
<tr>
<td>Tryptophan-bilirubin</td>
<td>24</td>
</tr>
<tr>
<td>PnA₁-bilirubin</td>
<td>40</td>
</tr>
<tr>
<td>PnA₂-bilirubin</td>
<td>37</td>
</tr>
<tr>
<td>PnA₃-bilirubin</td>
<td>40</td>
</tr>
<tr>
<td>PnA₄-bilirubin</td>
<td>35</td>
</tr>
</tbody>
</table>

---

**Table V**

**Binding Constants for Bilirubin and Human Serum Albumin**

Binding was quantified by the use of fluorescence quenching titrations at different protein concentrations as shown in Fig. 14. It is assumed that at concentrations greater than 6 X 10⁻⁶ the first 2 mol of cPnA are essentially all bound. As before, the nonlinear least squares routine was used, and three binding constants were required to produce accurate fits. The third binding constants were 8 X 10⁵, 7 X 10⁶, and 6 X 10⁵ at 5°C, 23°C, and 37°C, respectively. These constants are not reliable due to the absence of sufficient data points for r > 2. The RMS error is described in the legend to Table I. The enthalpy change calculated for the first binding constant from the 23°C and 37°C data is roughly 8.2 kcal/mol and the entropy change is roughly 9 cal/mol-degreete.
CONCLUSIONS

Binding and proximity relationships for fatty acids and bilirubin bound to human serum albumin were studied using absorption, circular dichroism, fluorescence enhancement, and fluorescence energy transfer techniques with conjugated linear polyene fatty acids. Electron paramagnetic resonance spectroscopy of nitroxide fatty acids and fluorescence quenching by nitroxide fatty acids were also used to determine proximity relationships. Binding and proximity relationships for bilirubin were monitored by fluorescence quenching. The data presented here generate the following picture of ligand binding to albumin. There are roughly six strong binding sites for fatty acids, with the first two fatty acids binding more strongly than the next four. The binding is very weakly temperature-dependent, indicating that the major driving force for binding is a positive entropy contribution. The first two fatty acids bind close together and further from the tryptophan residue than the third and fourth fatty acids. Taken together with the bovine serum albumin results of Sklar et al. (1977c), this supports the view that the first two binding sites are located in domain III, and the third and fourth fatty acids bind in domain II, which contain the tryptophan. Fluorescence quenching, EPR spectroscopy, and CD spectroscopy provide evidence that the first two fatty acids bind antiparallel and side by side. The first 2 mol of bilirubin bind a very large distance from the first two fatty acids. Fluorescence quenching data (not shown) and competition studies with linolenic acid indicate that the third fatty acid binds closer to the first two bilirubin sites, and that there is some competition between these fatty acids and bilirubin. Quenching studies indicate that the first two bilirubin sites are roughly equidistant from the first two fatty acid sites and from the tryptophanyl residue. Fluorescence quenching by bilirubin supports the notion that there are at least two strong binding sites whose affinities differ by an order of magnitude. Studies at different temperatures indicate moderate temperature dependence of binding affinity.

The relationships described here provide a set of approximate distances between ligands which follow the features of Brown's model and data on proteolytic fragments presented by Pederson and Foster (1969), Reed et al. (1975), and other workers. While the measurements depend on simplifying assumptions and the model for domain structures may be a simplification of the real state of affairs, a qualitative picture emerges which may be of heuristic value and also of clinical utility in describing drug-ligand interactions.

Acknowledgments—We have benefitted from helpful discussions with Drs. James R. Brown, Richard P. Wennburg, and Alonzo Ross. We thank Dr. Harden McConnell for use of his EPR spectrometer.

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Ligand Binding to Albumin

Absorption spectra were recorded using a Cary 14 spectrophotometer or a Cary 17 spectrophotometer.

The absorption characteristic was measured using a single-wavelength spectrophotometer in the laboratory of Dr. Reiner M"uller. Absorption spectra were scanned at various wavelengths of the Cary spectrophotometer and the Cary spectrophotometer was used to determine the absorbance values. The absorbance values were then plotted as a function of the wavelength to obtain the absorption spectrum. The absorbance values were then fitted to a theoretical model to obtain the absorption spectrum.

Figure 1. Absorption spectra of DNA in the absence and presence of DNA. The absorption spectrum of DNA in the absence of DNA is shown as a dotted line and the absorption spectrum of DNA in the presence of DNA is shown as a solid line. The absorption spectrum of DNA in the absence of DNA is shifted to the right compared to the absorption spectrum of DNA in the presence of DNA. This indicates that the presence of DNA increases the absorbance of DNA at the absorption wavelength.
Ligand Binding to Albumin

Figure 2. Spectral parameters for cesa and cesa bound to HSA. In each case, HSA (5 × 10^{-9} M) in 0.1 M sodium phosphate buffer, pH 7.4, is added to a 2 × 10^{-6} M solution of cesa fatty acid in the same buffer to give 0.01 M HSA and 0.05 M fatty acid. The excitation wavelength was 360 nm. The absorbance at the excitation wavelengths of the free fatty acid and fatty acid bound to HSA, respectively (see Fig. 1) as the fatty acid is bound to HSA.

Figure 3. Binding of cesa to HSA. Solutions of HSA (1 × 10^{-6} M to 1 × 10^{-5} M) were prepared using the buffer described in Materials and Methods. Cesa was added in ethanol in molar ratios ranging from 0.2 to 10. Final concentrations of cesa were always less than 0.25%. Relative fluorescence intensities give amount of cesa bound, and free concentrations were calculated by difference. For all but the highest molar ratios and concentrations, the linear least-squares concentrations were 10^{-7} M cesa to each data point. Similar results were observed for cesa 277 molar excess over HSA, and the results for cesa 1170 molar excess over HSA are shown in Table I.

Figure 4. Figure 9A shows FSR spectra of 12 NS, 10 NS, and 5 NS bound to HSA (1 × 10^{-6} M, 5 × 10^{-7} M, and 1 × 10^{-7} M, respectively). The linear least-squares curve for the data was calculated by the method described in Materials and Methods. The values of T and T', as well as the values of K and K', were calculated. Data is plotted in a bar chart format. The curves were calculated from the data presented in Table I. The least-squares adjusted values of the fluorescence maxima bound to HSA are shown in Table I.

Figure 5. Figure 11 shows FSR spectra of 12 NS, 10 NS, and 5 NS bound to HSA (1 × 10^{-6} M, 5 × 10^{-7} M, and 1 × 10^{-7} M, respectively). The linear least-squares curve for the data was calculated by the method described in Materials and Methods. The values of T and T', as well as the values of K and K', were calculated. Data is plotted in a bar chart format. The curves were calculated from the data presented in Table I. The least-squares adjusted values of the fluorescence maxima bound to HSA are shown in Table I.

Figure 6. Figure 12 shows FSR spectra of 12 NS, 10 NS, and 5 NS bound to HSA (1 × 10^{-6} M, 5 × 10^{-7} M, and 1 × 10^{-7} M, respectively). The linear least-squares curve for the data was calculated by the method described in Materials and Methods. The values of T and T', as well as the values of K and K', were calculated. Data is plotted in a bar chart format. The curves were calculated from the data presented in Table I. The least-squares adjusted values of the fluorescence maxima bound to HSA are shown in Table I.
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C B Berde, B S Hudson, R D Simoni and L A Sklar


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