The binding isotherms for the interaction of 5-doxyl stearic acid with bovine and human albumin

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Abstract Binding isotherms for the interaction of 5-doxyl stearic acid with bovine and human albumin are reported. The critical micelle concentration (CMC) and the limiting solubility of 5-doxyl stearic acid were determined using the electron spin resonance (ESR)–spin label method. The CMC and the limiting solubility of this spin-label stearic acid in saline–phosphate buffer are 3.5×10^{-5} M and 2×10^{-4} M, respectively. We found no ESR line width evidence for pre-association of the spin-label stearate below the CMC. Maximum binding of the spin-label stearate to both bovine and human albumin occurs before micelle formation. The binding isotherm for spin-label stearic acid interaction with bovine albumin is in agreement with data obtained by others using [1-^{14}C]stearic acid. For human albumin, comparison is difficult since previous data obtained with [1-^{14}C]stearic acid vary widely. Comparison of the ESR $2T_{\parallel}$ values (the splitting between low and high field extremes, a measure of the degree of immobilization of protein-bound spin-label stearate) for bovine and human albumin indicates a greater immobilization of the spin-label molecules bound to human albumin. The binding data indicate that complexes are formed with bound spin-label stearate/albumin ratios of at least 18. The computed equilibrium constants for both bovine and human albumin indicate that the first seven spin-label molecules are tightly bound, $\log K > 5.0$. The species predicted to form in solution by these equilibrium constants are reported.

Supplementary key words critical micelle concentration · electron spin resonance · equilibrium constants · binding sites · arginine · hydrophobic interactions

The binding isotherms reported for 1-^{14}C-labeled stearic acid interaction with bovine and human albumin differ significantly (1–3). A significant difference is also observed between the binding isotherms reported by Goodman (2) and Ashbrook et al. (3) for the interaction of [1-^{14}C]stearate with human albumin. These differences may be due in part to at least two factors. First, a larger correction was made for the effect of dimerization of stearic acid in the aqueous phase of human albumin (3) and, second, these differences may be induced by the procedure used to measure the binding isotherm (4, 5). The binding isotherms reported above were determined using the equilibrium partition method (1–6).

The technique of equilibrium partition analysis developed by Goodman (2, 6) for measuring the interaction of albumin with fatty acids involves the equilibrating of the fatty acid between two phases: *n*-heptane and aqueous solutions of albumin in buffer. The concentration of unbound fatty acid in the aqueous phase was determined from the fatty acid concentration in the *n*-heptane in the presence and absence of albumin in the aqueous phase (2, 6). The problems associated with this method, i.e., heptane binding to albumin causing perturbation effects of competitive binding and protein structural changes (4), have been discussed by many investigators (4–12). The equilibrium dialysis method has been reported to be unusable for fatty acids containing 12 or more carbon atoms in buffers at pH 7.4 (5). The question of whether stearic acid association occurs below the critical micelle concentration (CMC) remains unanswered (6); thus, the correction made for stearate dimerization in the aqueous phase may be unnecessary.

The ideal method to determine binding isotherms would be one that determines both bound and free ligand in the presence of albumin. In this communication, we describe the application of the electron spin resonance (ESR)–spin label method (13–20) to determine the binding isotherm of 5-doxyl stearate to bovine and human albumin. The ESR–spin label method has three distinct advantages over those described above: *1*) preparation of a protein-free solution is not

Abbreviations: CMC, critical micelle concentration; ESR, electron spin resonance.

necessary; *2*) the increase in albumin-bound spin label can be monitored independently of the unbound stearate (17–20); and *3*) when association occurs in the aqueous phase, the line width increases and the peak-to-peak amplitude decreases in the ESR spectrum. The unknown factor concerning the application of the nitroxide probe in binding studies is the effect, if any (19, 20), of the bulkiness of the probe causing stearic perturbation of its local environment. Our ESR results for spin label stearic acid binding to bovine albumin is in agreement with the stearate data of Spector, Fletcher, and Ashbrook (1). However, the binding isotherm for spin-label stearic acid to human albumin compares more favorably with the data of Goodman (2). No evidence for pre-association of spin label stearate below the CMC is observed in the ESR spectra.

EXPERIMENTS

Chemicals

The 5-doxyl stearic acid (2-[3-carboxypropyl]-4,4-dimethyl-2-tridecyl-3-oxazolidinyloxyl) was obtained from Syva, Palo Alto, CA. Saline–phosphate buffer (0.130 M NaCl, 0.020 M Na_2HPO_4 and 0.003 M KH_2PO_4, pH at 37°C of 7.4) was used as the solvent in all the experiments reported. Neither calcium nor magnesium was added to the buffer since it is well established that both of these ions are known to interact with albumin to form low solubility salts with fatty acids (21, 22). The reported solubility of calcium stearate in water is $2.7 \pm 1.7 \times 10^{-6}$M at 26.7°C and $1.0 \pm 0.6 \times 10^{-6}$M at 60°C (21); and the reported solubility of magnesium stearate is 1.18×10^{-4}M at 35°C (22). The highest purity reagents and deionized, doubly distilled water were used to prepare the buffer solutions. The deionized, doubly distilled water was reboiled just before use to remove dissolved CO_2. All solutions were prepared and stored under high-purity nitrogen.

Albumins

The crystalline human albumin, number A-1887, lot number 16C-7281, 16.2% nitrogen, fatty acid content less than 0.005%, was obtained from Sigma Chemical Co., St. Louis, MO. Cellulose acetate electrophoresis using barbital buffer, pH at 8.6, 0.075 ionic strength was run on this sample and only one peak was observed. The monomer, dimer, and polymer content of the human albumin sample were determined using the gel chromatographic technique. A solution of human albumin, 150 mg in 5 ml of 0.2 M NaCl–0.05 M Tris buffer, pH 8.0 at 4°C, was applied to a column (5.0 × 100 cm) packed with Sephadex superfine G-150 and equilibrated with the same buffer (19). The column was developed at 10.4 ml/hr with the saline–Tris buffer. Fractions containing 5.2 ml were collected and the absorbance was determined at 278 nm. Three peaks were observed representing 92% monomer, 6% dimers, and 2% polymers.

The crystalline fatty acid-free bovine albumin was obtained from Miles Laboratories, Inc., Elkhart, IN, code number 82-002, lot number 21, control C34-5, 98.7% protein by micro Kjeldahl analysis, and fatty acid content of 0.04 mol per mol of bovine albumin. Cellulose acetate electrophoresis using barbital buffer at pH 8.6, ionic strength 0.075 showed a single peak. Using the same saline–Tris buffer system described above and passing 150 mg of bovine albumin over the G-150 superfine column, the polymer content was found to be 3%, dimer 11%, and monomer 86%. No further purification of either albumin sample was undertaken. For all calculations made in this study we used 69,000 and 65,000 for the molecular weights of human and bovine albumin, respectively.

Instrumentation

The electron paramagnetic resonance spectra were recorded on a Varian E-12 spectrometer. This unit was equipped with a Varian variable temperature controller and sample Dewar. The temperature of the cavity was maintained at 37 ± 0.5°C using copper–constantin thermocouples with an ice junction coupled to a 8200A Fluka digital voltmeter. A Radiometer research pH meter model 64 coupled to a thermostated micro-electrode type CK2321C was used to measure the pH of the various solutions. A Gilford scanning spectrophotometer was used to record the spectrum of the protein solutions.

Critical micelle concentration

Various amounts of a stock solution of spin-label stearic acid in spectral grade chloroform ($\sim 1.1 \times 10^{-6}$ mol/ml) were added to variously sized volumetric flasks. The chloroform was removed using a gentle flow of high-purity nitrogen. Previous investigators have added the spin label as methanol or ethanol solutions without removal of the solvent, on the assumption that the alcohol did not influence the binding of the spin label to the protein, nor did it cause a configuration adaption in the protein (17–19). It is well known that the presence of these solvents alters the CMC of surfactants. For this reason, we decided to use the chloroform removal procedure. After the chloroform was removed, saline–phosphate buffer was added and the samples were equilibrated at 37 ± 0.5°C. The ESR spectra were measured using 10

mW power, modulation amplitude of 1.6 gauss, time constant of 3.0 sec, and a 16-min scan rate. The line widths measured at this modulation amplitude will be a few percent increased over the natural line width, as discussed by Smith (23), Smith (24) and Jost and Griffith (25). Samples were sealed in thin-wall capillaries and positioned in the Dewar unit using a quartz sleeve. All spectra were recorded at 37 ± 0.5°C. Only the receiver gain was varied to accomodate increases in peak amplitudes with increasing spin label concentration. All peak-to-peak amplitudes were then normalized to a receiver gain of 3.2×10^4. Samples were run in triplicate. Above spin label concentrations of 5×10^{-6}M, the coefficient of variation was 2% for the measured amplitudes. However, below this concentration (at binding ratios of stearate/albumin <1.0), the uncertainty increased with decreasing signal to noise ratio. Typical spectra are shown in **Fig. 1.** The peak-to-peak amplitudes, normalized to a receiver gain of 3.2×10^4, of resonance lines a_1, a_2, and a_3 are a function of spin label concentration. The peak-to-peak amplitude of a_3 as a function of spin label concentration is shown in **Fig. 2.** Three distinct regions are observed: A monomer region 0–3.5×10^{-5}M spin label, a micellar region, 3.5×10^{-5}M to 19.0×10^{-5}M, and a region above 19.0×10^{-5}M where the spin label is insoluble. The critical micelle concentration of the 5-doxyl stearate is 3.5×10^{-5}M. Values reported in the literature for the CMC of sodium stearate in CO_2-free water are 5×10^{-5}M (25°C) using the surface tension method (26) and 3×10^{-5}M (25°C) using the spectral dye method (26). Our value for the CMC of 5-doxyl stearate in saline–phosphate buffer at 37°C is in excellent agreement. It is well established

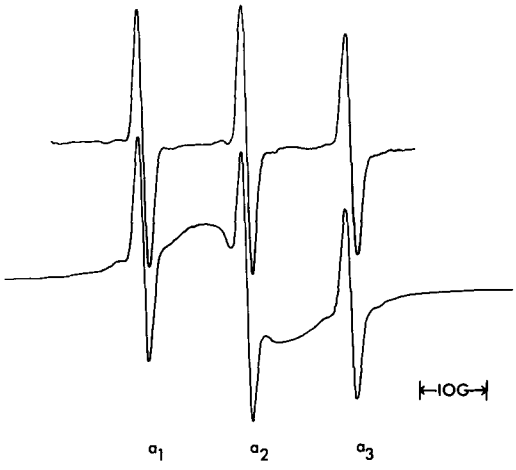

Fig. 1. Examples of the electron spin resonance spectra observed for 5-doxyl stearic acid in saline–phosphate buffer. The upper spectrum is obtained below the limiting solubility of spin-label stearic acid in this buffer system. The lower curve illustrates exchange-broadening in the micellar solution (25).

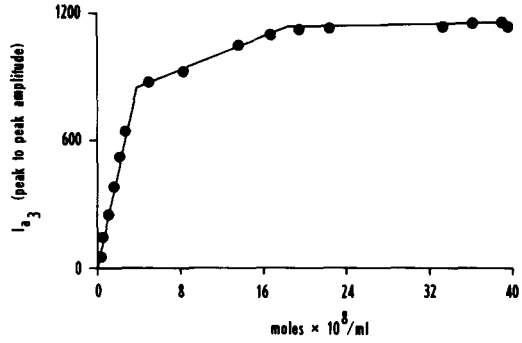

Fig. 2. Plot of the peak-to-peak amplitude of a_3 as a function of 5-doxyl stearate in saline–phosphate buffer. The first inflection seen in this plot is the CMC; the second occurs at the limiting solubility of the spin-label stearic acid in this buffer system.

that 0.1–0.2 M NaCl will reduce the CMC of surfactants, and an increase in temperature from 25°C to 37°C will increase the CMC.

Values for the limiting solubility of sodium stearate in water at pH > 9 as a function of temperature were reported by McBain, Bold, and Frick (27). Extrapolation of these data to a temperature of 37°C gives a limiting solubility of ~5×10^{-4}M. Our value for the limiting solubility of spin-label stearate in saline–phosphate buffer at 37°C of 2×10^{-4}M is in agreement. It is of interest to note that the limiting solubility of stearic acid in CO_2-free water at 25°C and 50°C is 2.1×10^{-6}M and 2.7×10^{-6}M, respectively (28).

In the monomer region (below 3.4×10^{-5}M) the ESR line width observed is 1.650 ± 0.025 gauss and the ratios of $a_1/a_2 = 0.952 \pm 0.006$, $a_3/a_2 = 0.798 \pm 0.023$, and $a_1/a_3 = 1.194 \pm 0.016$. In the micellar region (3.5×10^{-5}M to 19.0×10^{-5}M, respectively) the observed line width increased 1.700 ± 0.025 gauss, due to exchange broadening between the micelles and monomer, and the ratios of $a_1/a_2 = 0.905 \pm 0.010$, $a_3/a_2 = 0.798 \pm 0.023$, and $a_1/a_3 = 1.194 \pm 0.016$. Above 19.0×10^{-5}M the line width was 1.725 ± 0.070 gauss due to the exchange broadening between micelles, monomer, insoluble spin-label stearate, and the ratios of $a_1/a_2 = 0.840 \pm 0.027$, $a_3/a_2 = 0.716 \pm 0.013$, and $a_1/a_3 = 1.194 \pm 0.016$. The increase in line width is due to exchange broadening observed for spin label probes in micelles or insoluble aggregates (25).

Albumin–spin label samples

Various volumes of a stock solution (approximately 1×10^{-4}M) of spin label stearic acid dissolved in spectral grade chloroform were added to 2.0-ml volumetric flasks and the chloroform was removed by flowing a gentle stream of high-purity nitrogen into the flasks. After removal of the chloroform, 50 μl of a stock

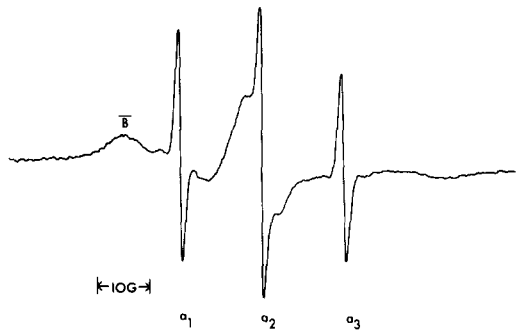

Fig. 3. An ESR spectrum recorded for the interaction of 5-doxyl stearic acid with albumin. *B* is the low field extreme due to albumin-bound immobilized spin label, and a_3 represents the unbound spin label.

solution of albumin ($\sim 2 \times 10^{-4}$M) dissolved in saline–phosphate buffer was added to each flask. These samples were then diluted to a total volume of 2.0 ml. All samples were stored under high-purity nitrogen and albumin stock solutions were prepared just before preparing samples. We found that equilibrium binding was obtained within 1 hr. After preparation all samples were kept cold at 4°C until 1 hr before spectral measurements were made; at that time they were equilibrated at 37 ± 0.5°C. This procedure was used to reduce the possibility of bacterial growth. A typical ESR spectrum for spin-label stearate bound to albumin is shown in **Fig. 3.** The low field resonance, *B*, is due to the spin label bound to albumin; all spectra were recorded in triplicate. Because the least effect of spin label interactions occurs to a_3 in the presence of albumin, we used this peak to determine the free concentration of spin label below the critical micelle concentration. The protein-bound (*B*) (low field resonance line) does not interfere with a_3, as shown in Fig. 3.

The free (C_f) and bound (C_B) spin-label stearate concentrations were computed in the following manner:

$$(I_u/I_s)\ (C_T) = C_f \qquad \text{Eq. 1}$$

$$C_T - C_f = C_B;\ C_B/A = \bar{n} \qquad \text{Eq. 2}$$

where C_T is the total concentration of stearate (mol/ml), A is the albumin concentration (mol/ml), I_u and I_s are the peak-to-peak amplitudes, normalized to a receiver gain of 3.2×10^4, of the unknown and standard, respectively, and $\bar{n}$ is the binding ratio.

RESULTS

Binding to bovine albumin

A Bjerrum "formation function" plot (29–31) is shown in **Fig. 4** for the binding of 5-doxyl stearic acid to bovine albumin using the spin label–ESR method. Also shown in Fig. 4 for comparison are equilibrium partition results for the binding of [I-^{14}C]-stearic acid to bovine albumin dissolved in saline–16 mM phosphate buffer containing 1.2 mM $MgSO_4$ at pH 7.4 and 37°C determined by Spector et al. (1). The spin-label stearic acid and [1-^{14}C]stearic acid bind to bovine albumin in a similar fashion. Note that values of $\bar{n}$ less than ~0.5 can be determined with greater precision by the radiotracer method for reasons discussed in the experimental section.

A maximum binding of spin-label stearic acid to bovine albumin occurs before the formation of spin-label stearate micelles, as shown in Fig. 4. This conclusion is further substantiated by the ESR data for bound spin label to bovine albumin shown in **Fig. 5.** Here we find that maximum binding of spin-label stearate to bovine albumin occurs before spin-label stearate micelles form. Quantitation of the data shown in Fig. 5 is questionable because I_B shifts upfield with increasing spin label binding, indicating greater mobility of the bovine albumin-bound spin-label stearate.

Binding to human albumin

A Bjerrum plot (29–31) is shown in **Fig. 6** for the binding of 5-doxyl stearic acid to human albumin. For comparison, the binding data reported by Goodman (2) and Ashbrook et al. (3) using the equilibrium partition method for [1-^{14}C]stearic acid to human albumin are also shown in this figure. Goodman (2) used a phosphate buffer of ionic strength 0.160 at a pH of 7.4 at 23°C; Ashbrook et al. (3) used the same buffer system, pH, and temperature reported by Spector et al. (1). Poor agreement exists between the data of Goodman (2) and Ashbrook et al. (3) as well

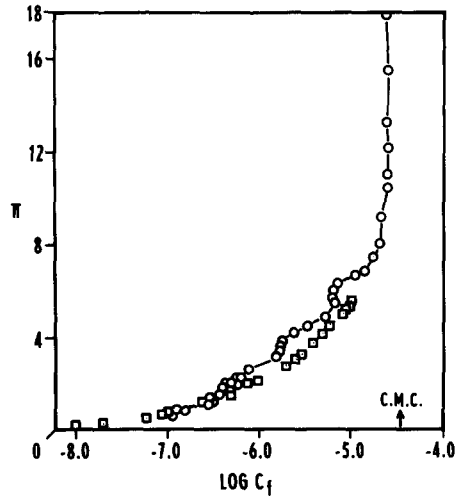

Fig. 4. A Bjerrum plot (29–31) of $\bar{n}$ vs. log C_f for the binding of 5-doxyl stearic acid to bovine albumin. The squares represent values reported by Spector et al. (1) for the binding of [1-^{14}C]-stearate to bovine albumin using the equilibrium partition method.

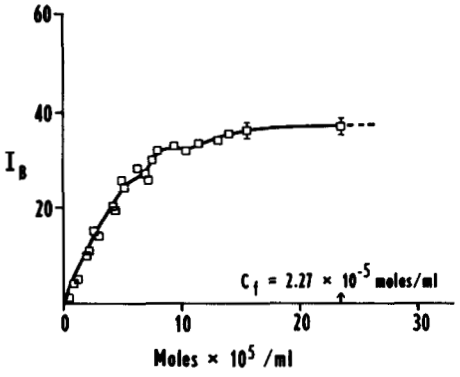

Fig. 5. A plot of the intensity of the bound spin label, B, is shown in the figure as a function of spin-label stearate bound to bovine albumin.

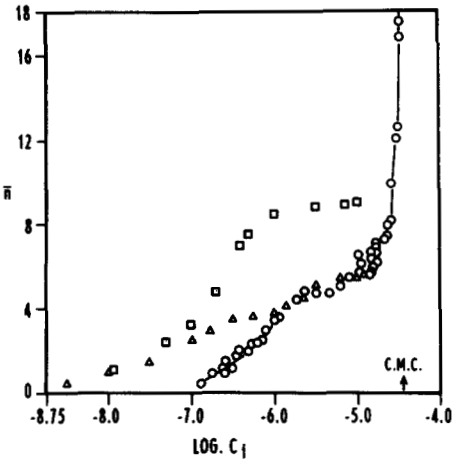

Fig. 6. A Bjerrum plot of $\bar{n}$ versus log C_f for the binding of 5-doxyl stearate to human albumin. Also shown are the data of Goodman (2) (triangles) and Ashbrook et al. (3) (squares) for the binding of [1-^{14}C]stearate to human albumin using the partition method.

as the spin label data for binding to human albumin. A possible explanation for these differences is proposed in the discussion section.

As in the case of spin label binding to bovine albumin, the maximum interaction of the spin-label stearic acid with human albumin occurs before micellization occurs as shown in **Figs. 6** and **7.**

Equilibrium constants

Equilibrium constants for the interaction of 5-doxyl stearic acid, S, with bovine and human albumin, P, were determined by a least squares fit of the $\bar{n}$ data (29–33).

$$\bar{n} = \frac{\sum_{i=1}^{n} iB_i(C_f)i}{1 + \sum_{i=1}^{n} B_i(C_f)^i},$$

where

$$B_i = K_1K_2 \ . \ . \ . \ K_n \qquad \text{Eq. 3}$$

In this technique, those equilibrium constants are found that best describe all the data by requiring the best fit to the error square sum U,

$$U = \sum (\bar{n}_{ej} - \bar{n}_{cj})^2 \qquad \text{Eq. 4}$$

where $\bar{n}_{ej}$ is the experimentally obtained value of $\bar{n}$ for each data point and $\bar{n}_{cj}$ is the $\bar{n}$ value calculated for that data point from the equilibrium constants which are thought to describe the interacting system. This requires that the condition

$$\frac{\partial U}{\partial K_j} = 0 \qquad \text{Eq. 5}$$

be satisfied for each equilibrium constant that describes the interaction of S with P, i.e.,

$$\begin{aligned} P + S &= PS, \quad K_1 \\ PS + S &= PS_2, \quad K_2 \\ PS_{n-1} + S &= PS_n, \quad K_n \end{aligned} \qquad \text{Eq. 6}$$

It should be noted that the above set of equations assumes that no protein association or dissociation reactions occur due to the spin-label stearate binding. This assumption was made since no data exist indicating that this is not the case. In addition, the reaction sequence assumes that any protein conformational changes which may occur do not affect the equilibrium constants for the binding of spin-label stearic acid molecules which were then associated with the protein prior to the conformational change. This assumption is necessary since the effect of conformational changes on $\bar{n}$ values cannot be measured. It should be noted that this technique treats all sites as independent. Finally, in these calculations, corrections for electrostatic interaction were not made and concentrations were used instead of activities.

Analysis of the data then involves solution of

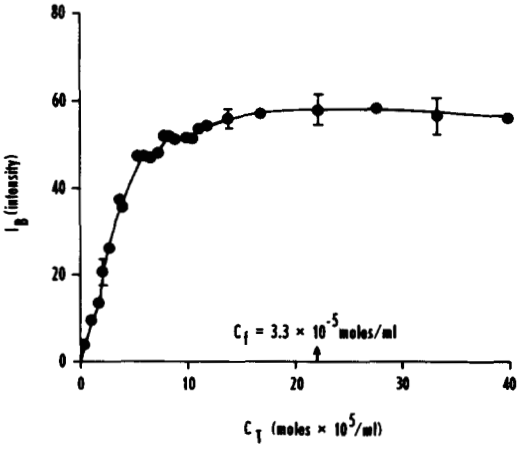

Fig. 7. A plot of the intensity of the immobilized spin label, B, as a function of spin-label stearate bound to human albumin.

TABLE 1. Equilibrium constants (log K) computed for the binding of 5-doxyl stearic acid to bovine and human albumin[a]

Reaction	Human		Bovine	
	Spin Label	[1-^{14}C]Stearate	Spin Label	[1-^{14}C]Stearate
$P + S = PS$	6.7	8.2		7.1
$PS + S = PS_2$	6.1	7.7		6.1
$P + 2S = PS_2$			13.0	
$PS_2 + S = PS_3$		7.3		5.8
$PS_2 + 2S = PS_4$	12.0	6.8	11.6	5.3
$PS_4 + S = PS_5$	5.3	6.7	5.2	5.2
$PS_5 + S = PS_6$	4.8	6.6	5.1	5.2
$PS_6 + S = PS_7$	5.2	6.5	5.4	4.8
$PS_7 + S = PS_8$	3.8	6.4	4.4	4.3
$PS_8 + S = PS_9$	4.2		4.1	
$PS_9 + S = PS_{10}$	4.3		4.2	
$PS_{10} + S = PS_{11}$	4.4		4.4	
Number of observations	39		48	

[a] The equilibrium constants reported previously (1, 3) for [1-^{14}C]stearate binding to these two albumins are also presented for comparison.

equation 5 for the $\bar{n}$ sets of reactions given by equation 6. As equation 5 is a nonlinear equation in the K_n values, this requires a nonlinear least-squares fitting program. We used a variable matrix method for minimization, VMM, previously described (32–33), and solved for the concentration of individual species using a matrix algebra algorithm computer program supplied to us by J. French, Kennecott Copper Corporation, Salt Lake City, UT. With this technique, values for $\log K_i, i = 1,2, \ldots n$ are assumed and values for $\frac{\partial U}{\partial K_i}$ and $\frac{\partial^2 U_2}{\partial K_i}$ are calculated and used to predict these K_i values which satisfy equation 5. These new K_i values are then used to calculate new values of $\frac{\partial U}{\partial K_i}$ and $\frac{\partial^2 U_2}{\partial K_i}$ and the process is repeated until equation 5 is satisfied. The program incorporates a random step routine to check for reconvergence to the same minima when equation 5 is satisfied. Moreover, it does not identify saddle points or other false minima. The data shown in Figs. 4–7 indicate that complexes are formed with S:P ratios of at least 18, so a value of $n = 18$ was used in equation 6. A least-squares treatment of all 18 equilibrium constants is impractical in terms of total computer time required. The data were fitted by selectively fitting blocks of 2, 3, 4, or 5 consecutive equilibrium constants while all other constants were assumed known, then using the obtained values as known constants while another block was fit. This process was repeated until a consistent fit to ±0.1 log unit for all constants was found. This required about 30 variations and 24 hr of computer (IBM 7030) processing time for each protein studied. The results obtained are given in **Table 1**.

It is interesting to note that the least-squares treatment of the data indicated that certain PS_i species do not form. For example, the species PS_3 consistently falls out of the least-squares fit for both the human and bovine albumin sample and the results indicate that, after the PS_2 species is formed, the next reaction that takes place involves the addition of two spin-label stearate molecules to form the PS_4 species. This same cooperativity effect is seen with the bovine albumin sample. In addition, the results indicate the PS species does not form with the bovine albumin and PS_2 is the first species formed. This result was consistently obtained for any K_i set chosen as the initial values. To test the possibility that this non-stepwise addition of spin label may be an artifact, ten additional points were obtained in the $\bar{n} \cong 1.0$ to 3.0 region using a slightly higher concentration of bovine and human albumin. The log K values then were redetermined by least-squares fit of the complete data set. In both cases, the reported non-stepwise addition behavior was obtained by least-squares fit of the data. It thus seems highly unlikely that this behavior is an artifact introduced by error in the data set. In addition, it should be pointed out that although values of $\bar{n}$ less than 1.0 have a large experimental uncertainly associated with them, the cooperative binding reported for the bovine albumin sample does require the fit of data of suitable accuracy in the $\bar{n} = 1.0$ to 2.0 region where the PS species would exist if the formation were stepwise.

For both albumins, the first seven spin-label stearic acid molecules are tightly bound. Binding of the eighth spin-label stearate molecule is much weaker, followed by a gradual increase in log K for the binding of subsequent ligands. This trend in log K is supported by the Bjerrum plots of the data. An increase in log K from 4.4 to 5.1 was computed for the formation of species PS_{12} to PS_{18}. This increase in log K above $i \sim 12$ may in part be an artifact due to lack of information on higher associated species which may be forming.

The species predicted to form in solution by these equilibrium constants are given in **Figs. 8** and **9.** Also plotted for information is the free spin-labeled stearic acid, S, as a percent of total protein; S, of course, continues to increase after the value of 100% total protein as additional spin-label stearate is added. Also given in the same figures is a comparison of the experimental and calculated $\bar{n}$ values, indicating the excellent fit to the experimental data.

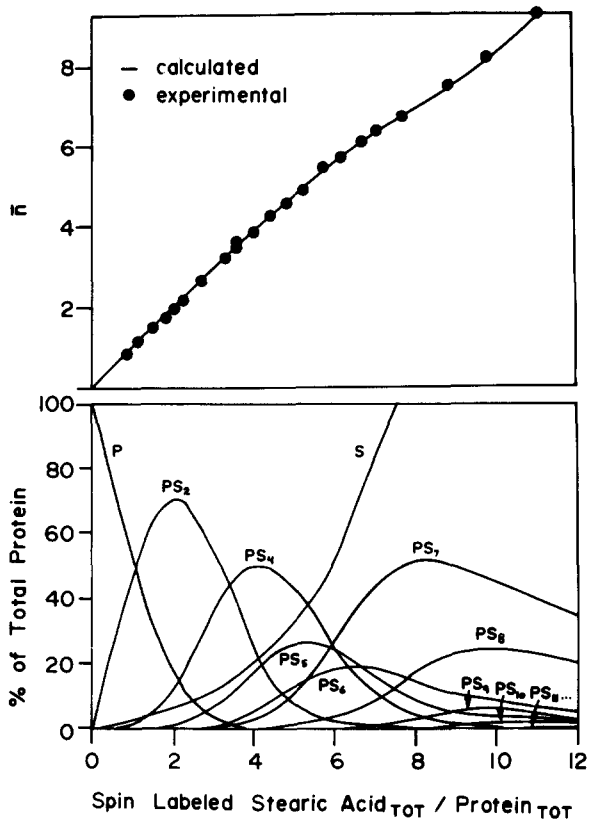

Fig. 8. A comparison of the experimental and calculated $\bar{n}$ values are shown in the upper figure for spin label binding to bovine albumin. The lower figure shows the species predicted to be formed in solution using the equilibrium constants given in Table 1.

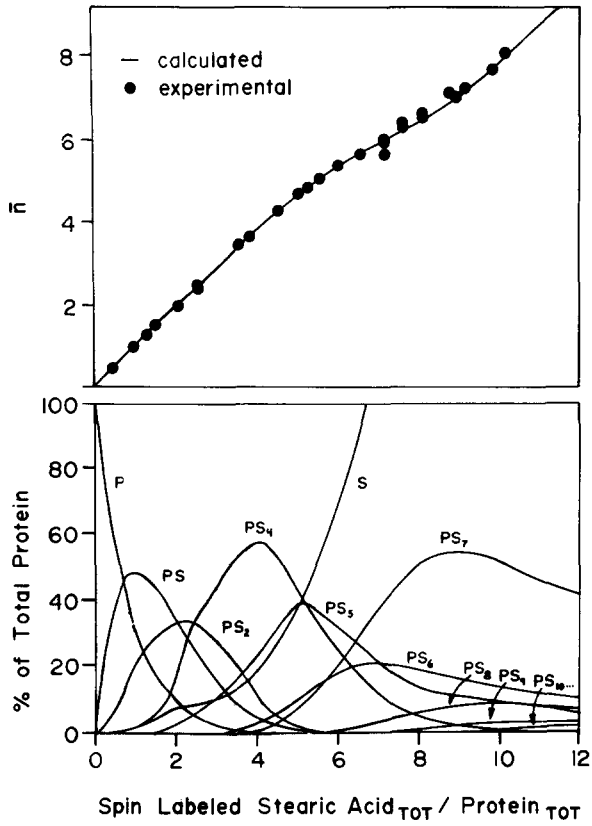

Fig. 9. A comparison of the experimental and calculated $\bar{n}$ values are shown in the upper figure for spin label binding to human albumin. The lower figure shows the species predicted to be formed in solution using the equilibrium constants given in Table 1.

Immobilized spin label

The binding of spin-label stearic acid to bovine and human albumin differs, as shown in Figs. 4–9. This difference was also apparent in the degree of immobilization of the spin label. The splitting between the low and high field extremes ($2T_{\parallel}$) is an index of the immobilization of the protein-bound spin-label stearate (13, 19), and increases as the motional freedom of the nitroxide decreases. A plot of $2T_{\parallel}$ for bovine and human albumin-bound spin-label stearate as a function of spin label concentration is shown in **Fig. 10.** Duplicate runs on different days agreed within ±1.0 gauss or less. It is apparent that the human albumin-bound spin label has the greatest decrease in motional freedom.

DISCUSSION

The linewidths of a_3 (unbound spin label) below the CMC, in the presence and absence of albumin, were the same. Based on this observation, we conclude that pre-association of the spin label does not occur below the CMC. Other investigators using the electrical conductivity or spectral methods also have reported finding no evidence of pre-association of fatty acid salts or other surfactants below the CMC (34–37). Thus corrections made for pre-association of fatty acids below the CMC in protein binding studies (1–3) appear to be unnecessary. It is also of some interest to point out that hydrocarbons, e.g., benzene, dissolved in water, do not show any evidence of pre-association (38, 39).

The binding isotherms for the interaction of 5-doxyl

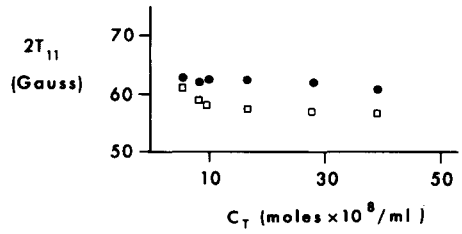

Fig. 10. Values of $2T_{\parallel}$ as a function of spin label concentration for spin label binding to bovine and human albumin. The squares are the values for bovine albumin.

stearic acid and [1-^{14}C]stearic acid (data of Spector et al. [1]) with bovine albumin are comparable. Our results suggest that a cooperativity in binding occurs in the $\bar{n}$ range of 1–4 (see Table 1) whereas the results of Spector et al. (1) suggest no cooperative binding of [1-^{14}C]stearic acid to bovine albumin in this range of $\bar{n}$.

On the other hand, binding of 5-doxyl stearic acid and [1-^{14}C]stearic acid to human albumin differed significantly. As shown in Fig. 6, not only is a significant difference observed between the binding isotherms for spin-label stearic acid and [1-^{14}C]stearic acid interaction with human albumin but also between the binding isotherms reported by Goodman (2) and Ashbrook et al. (3) for [1-^{14}C]stearic acid to human albumin. The reasons for this poor agreement may involve differences in buffer composition (1–3), albumin source (1–3), method of albumin purification (1–3), heptane purity (39), or failure to exclude CO_2 (28). Ashbrook and co-workers (3) included 1.0 mM $MgSO_4$ in their saline–phosphate buffer which may influence the binding results, as suggested in our experimental section. The results presented here do suggest that 5-doxyl spin-label stearic acid and [1-^{14}C]-stearic acid bind at similar sites on bovine and human albumin.

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