Binding of long-chain fatty acids to bovine serum albumin

ARTHUR A. SPECTOR,* KATHRYN JOHN, and JOHN E. FLETCHER

Laboratory of Metabolism, National Heart Institute, and Laboratory of Applied Studies, Division of Computer Research Technology, National Institutes of Health, Bethesda, Maryland 20014

ABSTRACT We have studied the binding of long-chain free fatty acids (FFA) to crystalline bovine serum albumin (BSA) that had been extracted with charcoal to remove endogenous fatty acids. The data were analyzed in terms of a model consisting of six high-energy binding sites and a large number of weak binding sites.

The high-energy sites were resolved into two distinct classes, each containing three sites. At 37°C and pH 7.4, k'_1 (the apparent association constant of a class of binding sites) was about 10^6 M^{-1} for binding to the three primary sites, and k'_2 was about 10^5 M^{-1} for binding to the three secondary sites. The number of weak (tertiary) sites was estimated to be 63 with a k'_3 of 10^3 M^{-1}. In general, palmitate and palmitoleate were bound more tightly than oleate, linoleate, stearate, or myristate, and much more tightly than laurate.

The association of palmitate with human and rabbit albumin also was analyzed in terms of this model. Palmitate was bound less firmly by human or rabbit albumin than by BSA. Palmitate binding to BSA was dependent upon the pH and temperature of the incubation medium.

Long-chain hydrocarbons that did not contain a free carboxyl group (methyl palmitate, cetyl alcohol, and hexadecane) were bound to a limited extent and weakly. The presence of positively charged protein sites and native protein tertiary structure were required for maximal binding of palmitate to BSA. Of nine other proteins tested, only β-lactoglobulin exhibited a significant capacity to bind palmitate.

SUPPLEMENTARY KEY WORDS free fatty acids · association constants · pH · temperature · hexadecane · methyl palmitate · cetyl alcohol · β-lactoglobulin

AN IMPORTANT FUNCTION of serum albumin is to bind long-chain fatty acids and thereby make them more soluble in aqueous solutions. This is of physiological significance, for albumin binds almost all of the free fatty acid (FFA) that is released into the blood from adipose cells. As such, albumin is the major vehicle for FFA transport through the plasma (2, 3). In the presence of albumin, the concentration of a long-chain fatty acid can be increased as much as 500 times above its maximum solubility in salt solutions of a composition like that of plasma. The interaction between the commonly occurring long-chain fatty acids and human serum albumin (HSA) has been studied in detail (4, 5). Considerable information is also available concerning the binding of detergents, dyes, and short-chain fatty acids to bovine serum albumin (BSA) (6). However, few quantitative data are available concerning the binding of the physiologically important long-chain FFA to BSA, the protein that is used almost exclusively in experimental work as the FFA carrier or acceptor. This communication concerns the mechanisms and quantitative aspects of the long-chain FFA–BSA association and the effect on this of some commonly employed variations in experimental conditions. In addition, we have compared the binding of palmitic acid to bovine, human, and rabbit serum albumin (RSA).

EXPERIMENTAL PROCEDURE

Materials

Unlabeled fatty acids, methyl esters, and cetyl alcohol were purchased from The Hormel Institute (Austin,

A preliminary report of this work was presented to the Council on Arteriosclerosis of the American Heart Association in San Francisco, California, October 19, 1967, and has appeared in abstract form (1).

Abbreviations: FFA, long-chain unesterified fatty acid or acids; HSA, crystalline human serum albumin; BSA, crystalline bovine serum albumin; RSA, crystalline rabbit serum albumin; $\bar{\nu}$, the average number of moles of bound FFA per mole of albumin; c, the molar concentration of unbound FFA in equilibrium with that bound to albumin; n, the number of individual sites in a given class of binding sites; k', the apparent association constant of a given class of binding sites.

* Present address: Departments of Medicine and Biochemistry, College of Medicine, University of Iowa, Iowa City, Iowa 52240.

Minn.). Hexadecane was obtained from Eastman Organic Chemicals (Rochester, N.Y.). Palmitate-1-^{14}C, palmitate-9,10-^{3}H, myristate-1-^{14}C, oleate-1-^{14}C, and cetyl alcohol-1-^{14}C were supplied by New England Nuclear Corp. (Boston, Mass.); *n*-hexadecane-1-^{14}C and linoleate-1-^{14}C, by Nuclear-Chicago Corporation (Des Plaines, Ill.). Methyl palmitate-1-^{14}C was synthesized from palmitate-1-^{14}C and methanol containing 14% BF_3 (7). Each radioactive compound was dissolved in *n*-heptane and extracted with alkaline ethanol (8). The ethanol was acidified and fatty acids were re-extracted into fresh heptane. Heptane (50 ml) containing the radioactive compound then was extracted with an equal volume of 0.01 N H_2SO_4 in a separatory funnel to insure removal of any remaining traces of water-soluble radioactive impurities, and unlabeled carrier fatty acid (10–20 μeq/ml) was added. Analysis of the purified compounds by thin-layer chromatography (8) indicated a radiopurity in fatty acid of at least 97%. Analysis of the heptane solutions of ^{14}C-labeled fatty acids by gas–liquid chromatography was made as before (9) except that the column effluent was collected in tubes packed with fine glass beads by means of a Packard Tri-Carb gas chromatography fraction collector. The following radiopurities were found: palmitate 97%, myristate 98%, stearate 95%, palmitoleate 95%, oleate 99%, and linoleate 94%. The radioactive impurities were contained in other long-chain FFA. It is likely that the true radiopurities are higher than these values, for samples that were initially purified by gas chromatography appeared to be only 98–99% radiopure by this analytical technique.

Crystalline HSA, BSA, and RSA were purchased from Pentex, Inc. (Kankakee, Ill.). Crystalline BSA also was obtained from Armour Pharmaceutical Co. (Kankakee, Ill.) and Nutritional Biochemicals Corporation (Cleveland, Ohio). Inherent fatty acids were removed by incubation of acidified aqueous solutions of these proteins with charcoal (Darco, Atlas Chemical Industries Inc., Wilmington, Del.)[1] according to the method of Chen (10). Additional purification steps were included as follows. The albumin solution was centrifuged at 5°C for 45 min at 100,000 *g*. After neutralization with NaOH, the supernatant solution was dialyzed at 4°C against 4 liters of distilled water for a minimum of 18 hr, the water being changed at least once during this time. Essentially all of the endogenous fatty acid as measured by titration (10) was removed by this procedure. Albumin concentration was determined from the absorbance of the solution at 280 mμ. A dried sample of each albumin preparation served as the standard, and molar concentrations were calculated by using a molecular weight of 66,000. Electrophoretic analysis of typical BSA and HSA preparations were made on standard microscope slides coated with 2% special Noble agar and stained with SF light green (11). These "FFA-free" proteins migrated as a single band in this system. Streptomycin (1 mg/10 ml) was added to most of the albumin preparations before they were incubated.

Unless noted otherwise, a salt solution containing 0.116 M NaCl, 0.0049 M KCl, 0.0012 M $MgSO_4$, and 0.016 M sodium phosphate, pH 7.4, was used for all incubations. This is referred to in the text as "phosphate-buffered salt solution."

Preparation of Chemically Modified BSA

BSA was acetylated with acetic anhydride at both pH 5 and 8 by the method of Fraenkel-Conrat, Bean, and Lineweaver (12). The protein was incubated with 0.5 M *O*-methylisourea sulfate by the method of Tabachnick (13). BSA was allowed to react with formaldehyde according to the procedure of Teresi (14). These protein solutions were then dialyzed for at least 48 hr against 4 liters of distilled water, the dialyzing solution being changed at least three times during this period. Free amino groups were measured by the ninhydrin test (15) with native BSA as the standard. Protein concentration in these chemically modified preparations of BSA was determined by the biuret method (16).

Incubation Flasks

Special flasks were constructed from screw-capped glass tubes (Corning, Pyrex, No. 9825). These tubes were cut to an over-all length of 5.8 cm, and the bottom was closed with a flat seal. The I.D. was 1.3 cm. A hollow glass sampling tube, 2.6 cm long and 0.6 cm O.D., was mounted in the center of the flask and 0.25 cm above the base. A tightly fitted glass rod, 3.2 cm long and 0.32 cm in diameter, was inserted into the sampling tube and allowed to rest on the bottom of the flask. When 2 ml of fluid was added to these flasks with the glass rods in place, the fluid level was at least 1 cm below the upper orifice of the sampling tube, and the liquid did not touch this orifice during to-and-fro shaking in an incubator. Sufficient clearance existed between the sampling tube and walls of the flask to permit addition of the reagents by pipet without touching the glass surfaces. The flasks were sealed with Teflon-lined screw caps.

The rationale for constructing these flasks was as follows. We wished to use concentrations of albumin in the physiological range. As such, it was necessary to work with small volumes of solutions in order to conserve reagents. In addition, the contents of the incubation

[1] Only Darco was used in this work to remove fatty acids from albumin. However, as reported by Chen (10), several other commercially available activated carbon preparations can be substituted for Darco in this procedure.

flask had to be shaken at a constant temperature, particularly at 37°C. The only practical way to do this was to use a vessel that could be immersed in a temperature-controlled water bath incubator. We chose a short, screw-capped tube with a Teflon-lined cap in order to prevent large losses of organic solvent due to evaporation. The reaction mixtures were prepared by layering the organic phase containing radioactive material over the aqueous phase. After incubation, the lower phase (aqueous) had to be sampled. When no protein was present 1% or less of the total radioactivity was contained in the aqueous phase at equilibrium. In these cases, even minimal contamination of the pipettes used to sample the aqueous phase would have produced large errors. The presence of the sampling tube permitted us to insert a pipette directly into the aqueous phase without touching the overlying organic solvent.

Initial incubations were performed without insertion of a glass plug inside the sampling tube. About one-half of the 1 ml aqueous phase was present inside the sampling tube during these incubations, and equilibrium occurred very slowly. When a glass plug was placed inside the sampling tube, almost all of the aqueous phase was excluded from the tube during incubation, and equilibration occurred much more quickly. The possibility of error caused by binding of fatty acid to the inner surface of the sampling tube when the glass plug was removed was investigated in the following way: the equilibrium distribution of palmitate-1-^{14}C between *n*-heptane and phosphate-buffered salt solution was determined both in these incubation flasks and in small separatory funnels similar to those used by Goodman (4). The glass outflow stems were cut off from the separatory funnels before use. Incubation was done at room temperature, the funnels being agitated with a Burrell "wrist-action" shaker. After incubation, a small glass vial was used to collect the aqueous phase from each separatory funnel. The vial was washed with two aliquots of the aqueous phase, and the pipette that was to be used was also rinsed twice with the aqueous solution. An additional portion of the aqueous phase then was collected in the vial, and an aliquot of the solution was taken by means of the washed pipette for isotope counting. Aliquots of the aqueous and heptane phases were taken from our incubation flasks as described below. At a given concentration of palmitate-1-^{14}C in heptane, the concentration in the aqueous phase obtained from our incubation flask was either the same or slightly larger than that in the aqueous phase obtained from the separatory funnel. The recovery of radioactivity from the flasks ranged from 97.4 to 102.3%. Hence, the present method for measurement of the FFA partition ratio appears to be at least as accurate as that employed by Goodman (4). However, the possibility remains that small errors of similar magnitude occur in *both* his and our procedures because of loss of fatty acid from the protein-free aqueous phase.

A second type of incubation flask was constructed so that two different aqueous solutions could be incubated simultaneously with the same organic phase. The flask height was 4 cm and the I.D. was 2.7 cm. A glass partition extending 1.7 cm above the base separated the lower part of the flask into two compartments. One sampling tube extended into each compartment. Aqueous solution (3 ml) was placed into each compartment after the glass rods were inserted into the sampling tubes. Preliminary tests of each flask with dye solutions demonstrated that mixing of the two solutions by splashing over the glass partition during prolonged to-and-fro shaking did not occur. Heptane was added to a level of 0.5 cm above the glass partition (approximately 6 ml). In this way, both compartments containing aqueous solutions were in contact with a continuous heptane phase, and the level of heptane was low enough so as not to touch the upper orifice of the sampling tubes during shaking. The flasks were sealed with a Teflon stopper.

Incubation and Analysis

The procedure we used was a modification of the equilibrium partition method described by Goodman (4). In most experiments, approximately 30 single-compartment flasks were prepared, 15 containing only buffer solution and 15 containing protein, usually albumin, dissolved in the buffer solution. The removable glass rods were inserted before the solutions were added to the flasks. Buffer solution (0.5 ml) of twice the desired final concentration was added to all flasks, followed by 0.5 ml of distilled water to one group and 0.5 ml of albumin in distilled water to the other group. Next, heptane containing the required amount of labeled ligand of known specific radioactivity was added, and the total volume of heptane in each flask was made up to 1 ml. The range of ligand concentrations initially present in the heptane phases was 1×10^{-5} M to 5×10^{-2} M. A single albumin concentration was used in each experiment, this usually being 3.6×10^{-4}M. When unsaturated fatty acids were used, the flasks were gassed with N_2; with other ligands, air served as the gas phase. The flasks were capped tightly and incubated with shaking in a constant temperature water bath.

After incubation, samples of the heptane phase were taken for analysis with a micropipet. Next, the glass rod was removed with forceps so that a portion of the aqueous phase filled the sampling tube. A micropipet was inserted through the tube and rinsed twice with liquid from the aqueous phase; a sample of the aqueous solution was then taken for analysis.

Aliquots of the heptane and aqueous phases from each flask were added to 18 ml of a toluene–methanol scintillator solution (70:30 v/v) containing 0.3% 2,5-diphenyloxazole and 0.01% 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene. A Packard Tri-Carb liquid scintillation spectrometer was used for measurements of radioactivity. Corrections for quenching were made by counting the samples again after addition of internal standard. Quenching occurred when albumin was present in the aqueous samples, but it did not exceed 2.5% in any experiment.

The reproducibility of results obtained by this method is shown in Table 1. A single pair of flasks was used in the first experiment. One flask contained heptane and buffer; the second contained heptane and buffer–albumin. Each of the heptane and aqueous phases was sampled eight times, and the radioactivity present in each sample was determined. Eight pairs of flasks were incubated in the second experiment. The heptane and aqueous phases of each flask were sampled only once, and the radioactivity in each sample was measured. In both experiments, the fractional standard error of the measured radioactivity contained in each phase was 1.5% or less.

The time required to reach equilibrium in this system was determined in preliminary studies. Table 2 contains data from an experiment in which BSA containing palmitate-9,10-^{3}H was incubated at 37°C with heptane containing palmitate-1-^{14}C. During the course of this incubation, palmitate mass in the aqueous phase increased by 22%. After 7 hr, the aqueous phase contained approximately the same fraction of the total palmitate-1-^{14}C and of the total palmitate-9,10-^{3}H that was present initially in the system, namely 54–59% of each isotope. This distribution was maintained until the experiment was ended at 24 hr. Equilibrium was reached within 36 hr at 23°C and 144 hr at 4°C. In separate experiments, it was determined that the partition of radioactive FFA between the heptane and albumin-free buffer phases also reached the equilibrium point at these times. For convenience, we used an overnight incubation (16 hr) at 37°C and a 40 hr incubation at 23°C.

TABLE 1 REPRODUCIBILITY OF RESULTS OBTAINED WITH PALMITATE-1-^{14}C BY EQUILIBRIUM PARTITION ANALYSIS

Sample	Number of Determinations	Radioactivity	Fractional Standard Error
		cpm	%
One pair of flasks			
Heptane I	8	19,900	0.60
Buffer*	8	279	1.5
Heptane II	8	1,270	1.0
Albumin†	8	21,600	0.30
Eight pairs of flasks			
Heptane I	8	39,500	0.40
Buffer	8	567	0.80
Heptane II	8	2,360	1.2
Albumin	8	42,800	0.80

The flasks were incubated for 18 hr at 37°C.
* Phosphate-buffered salt solution.
† Bovine serum albumin.

TABLE 2 EQUILIBRATION BETWEEN PALMITATE-1-^{14}C IN *n*-HEPTANE AND PALMITATE-9,10-^{3}H IN THE AQUEOUS PHASE CONTAINING ALBUMIN

Time of Incubation	Radioactivity Contained in the Aqueous Phase	
	Fraction of Total ^{14}C	Fraction of Total ^{3}H
hr	%	
0	0	100
0.5	24	84
1	49	62
2	50	66
4	59	62
7	58	59
15	57	56
18	58	54
24	59	58

At the start of incubation, each of nine separate flasks contained 0.345 μmole of BSA plus 1.04 μeq of palmitate-9,10-^{3}H (268,700 cpm/μeq) in 1 ml of phosphate-buffered salt solution and 1.20 μeq of palmitate-1-^{14}C (96,500 cpm/μeq) in 1 ml of *n*-heptane. The incubation was done at 37°C. Flasks were removed, and the aqueous and heptane phases were analyzed at the indicated time points.

Both the aqueous and heptane phases remained clear throughout the incubation, and precipitates did not form at the interface. To test for more subtle structural changes in the protein, we examined the following properties of both unincubated samples of albumin and those incubated without added FFA: (*a*) light absorption spectrum in the presence of 2-[(4′-hydroxyphenyl)-azo]benzoic acid (17); (*b*) fluorescence spectrum after addition of 8-anilino-naphthalene-1-sulfonic acid (9); (*c*) ultraviolet fluorescence spectrum after excitation at 280 mμ (18); and (*d*) ultraviolet light absorption spectrum. In each case, results with samples of incubated albumin were almost identical with those with unincubated samples. While these data do not completely exclude the possibility that structural changes occurred in the incubated albumins, they suggest that such changes, if they occurred at all, were small. A similar conclusion was reached by Goodman concerning his system (4).

Calculations

The total quantity of FFA present in both the heptane and aqueous phases was calculated from the radioactivity measurements. Concentrations rather than activities were used in subsequent calculations. A partition ratio was determined for each of the flasks containing no

albumin (4, 19). This was defined as the total amount of FFA in heptane (1 ml) divided by the total amount in the aqueous phase (1 ml) at equilibrium. As was observed previously (19), the partition ratio varied as a function of the total concentration of FFA in the heptane phase. A graphic plot of the partition ratio against the molar concentration of FFA in heptane was made, the latter being expressed as the negative logarithm (19). Using this graphic plot and the measured concentration of FFA in the heptane phase of those flasks containing albumin, we determined the molar concentration of *unbound* FFA (c). This is possible because two aqueous solutions (one with and one without albumin) that are in equilibrium with the same concentration of FFA in heptane are, in turn, in equilibrium with each other. Next, we calculated the concentration of *bound* FFA in the aqueous phases containing albumin by subtracting the unbound from the total concentration, and the average number of moles of FFA bound per mole of albumin ($\bar{\nu}$) was determined.

The data consisting of experimental values of $\bar{\nu}$ for each value of c was fitted to a model of the form (20):

$$\bar{\nu} = \sum_{i=1}^{j} \frac{n_i k'_i c}{1 + k'_i c} \qquad \text{(Eq. 1)}$$

where j = number of distinct classes of binding sites;
k'_i = apparent association constant for class of sites i;
n_i = number of sites in class i.

The computer programs and mathematics involved employ a nonlinear, least-squares fitting method based on an eigenvalue–eigenvector search method to reduce the sum of squares. The basic programs are catalogued for the IBM 360/50 computer system. A curve-fitting procedure that applies to these binding data has been described in detail (21). The definitions $n_i k_i = P_i$, and $k_i = P_{i+1}$ were made. Equation 1 thus becomes:

$$\bar{\nu} = \sum_{i=1}^{j} \frac{P_i c}{1 + P_{i+1} c}. \qquad \text{(Eq. 2)}$$

Using the theory of least squares, we obtained estimates of P_i and P_{i+1} for a fixed number of classes of binding sites, j. An estimate for each n_i is $n_i = P_i/P_{i+1}$, and an estimate for the corresponding constant is $k'_i = P_{i+1}$. We arbitrarily rounded P_i/P_{i+1} to the nearest integer for an initial trial value. Successive trials were made for each of $n_i + 1$, n_i, and $n_i - 1$, for each i. Once the selection of a trial value of each n_i was made, the corresponding k'_i was adjusted by the same methods (holding n_i fixed) to give a least-squares fit of the data. The best model was selected based upon *consistency* of fitting of *each* set of data, minimum sum of squares, and distribution of the uncertainties in the derived parameters. The parameter uncertainties are similar to "standard errors" for a linear regression model. However, the model used here is nonlinear, and the parameter uncertainties can be interpreted only as asymptotic estimates of the standard error.

Corrections for electrostatic interactions were not applied, and no attempt was made to relate apparent association constants obtained in these incubation media to the intrinsic association constants of the albumins.

Celite Experiments

Radioactive lipids were adsorbed on Celite 545 by the method of Avigan (22). 1–150 mg of Celite containing 0.1 μmole of lipid per mg was incubated with shaking at either 23 or 37°C with 3 ml of 0.1 mM albumin in phosphate-buffered salt solution. Flasks (25 ml) sealed with rubber serum stoppers were used. The maximum uptake of labeled lipid by albumin occurred within 24 hr, and this incubation time was used routinely. Additional incubations in which no protein was present in the buffer solution were done simultaneously in stoppered conical centrifuge tubes. After incubation, the Celite was sedimented by centrifugation at room temperature for 3 min at 2000 g, and the radioactivity contained in the supernatant solution was determined.

RESULTS

FFA and Bovine Albumin

Data obtained for oleic acid binding to BSA at 37°C in phosphate-buffered salt solution are shown in Fig. 1.

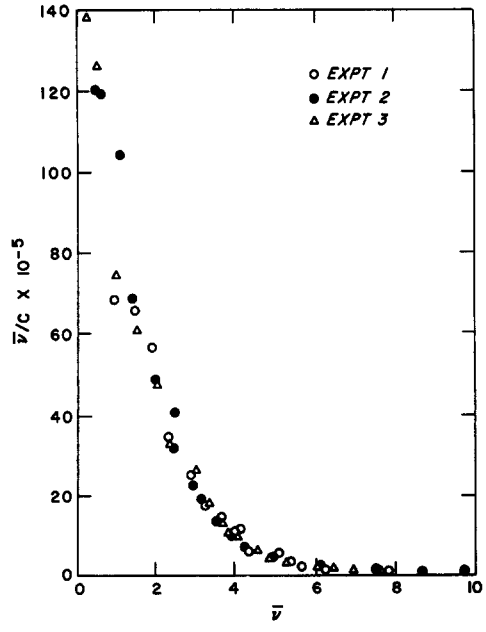

FIG. 1. Scatchard plot of data for binding of oleic acid-1-^{14}C to crystalline bovine serum albumin at 37°C in phosphate-buffered salt solution, pH 7.4.

In this graph, $\bar{\nu}$ is plotted against $\bar{\nu}/c$ according to the method of Scatchard (20). The results from three separate experiments are in close agreement. The points do not indicate a linear correlation, which suggests that BSA, like HSA (4), contains more than one class of oleate binding sites. In additional experiments, we observed that a maximum of 13.5 μeq of oleate could be bound by 1 μmole of BSA in this system. Results similar to these were obtained when palmitic, palmitoleic, myristic, stearic, linoleic, and lauric acids were incubated with BSA at 37°C in phosphate-buffered salt solution. However, the maximum $\bar{\nu}$ value that could be obtained with these acids was lower, varying from 8.4 with laurate to 6.5 with stearate. Similar values for the maximum $\bar{\nu}$ were obtained from incubations with FFA adsorbed on Celite.

An analysis of the binding data was made in terms of three separate classes of BSA binding sites (Table 3). The number of third-class binding sites could not be precisely determined. A value of 63 was chosen because this was best for oleate, the acid for which we had the widest range of data. This value also was compatible with the data obtained with each of the other acids. The presence of six higher-energy sites was established for each acid. These higher-energy sites, except when laurate served as the ligand, could be separated into two distinct classes, each containing three sites. The values of k' for FFA binding to the three primary sites are 10 times as large as those for binding to the three secondary sites and 1000 times as large as those for binding to the tertiary sites. With laurate, the final values obtained for k'_1 and k'_2 in the three-class model were identical, and the initial computer trials indicated that the k' values for the primary and secondary sites were not statistically different (correlation = 0.995). Hence, for laurate binding, the six higher-energy sites are considered as a single class. The laurate analysis in terms of the three-class model also is listed in Table 3 in order to facilitate comparisons between lauric and the other acids.

Fig. 2 shows plots of the data for each acid and the binding curves derived from the values of n and k' listed in Table 3. These are graphs of $\bar{\nu}$ against the negative logarithm of c. In general, palmitate and palmitoleate were bound more tightly by BSA than myristate, oleate, linoleate, or stearate and considerably more tightly than laurate.

Constants for the association of palmitate with HSA and RSA also are listed in Table 3. The theoretical binding curves constructed from these values of n and k' are shown in Fig. 3. These data were analyzed adequately by using the same three-class model that was developed for BSA. The number of HSA and RSA tertiary binding sites also could not be precisely determined. However, a lower root-mean-square error was obtained when 50 or more tertiary sites were used as compared with 20 or less. The value of 63 was used so that data obtained with each of the three albumins might be compared. If one uses our data and a "2,4,20" model, the values of k' for palmitate binding to HSA at 23°C are similar to those obtained by Goodman (4). However, the "3,3,63" model produced a slightly better

TABLE 3 CONSTANTS FOR THE BINDING OF FFA TO SERUM ALBUMINS IN PHOSPHATE-BUFFERED SALT SOLUTION

Fatty Acid	Temperature	Number of Data Points	Primary Sites		Secondary Sites		Tertiary Sites		Root-Mean-Square Error*
			n_1	$k_1 \times 10^{-6}$	n_2	$k_2' \times 10^{-5}$	n_3	$k_3' \times 10^{-3}$	
	°C			M^{-1}		M^{-1}		M^{-1}	
Bovine Albumin									
Palmitic	23	54	3	28.80 ± 2.10†	3	5.98 ± 0.49	63	0.66 ± 0.016	0.21
Palmitic	37	86	3	6.78 ± 0.42	3	5.00 ± 0.33	63	0.96 ± 0.024	0.23
Palmitoleic	37	30	3	5.97 ± 0.87	3	8.61 ± 1.3	63	0.99 ± 0.014	0.26
Oleic	37	50	3	3.96 ± 0.25	3	1.26 ± 0.060	63	0.50 ± 0.030	0.16
Linoleic	37	23	3	2.98 ± 0.19	3	1.62 ± 0.090	63	0.11 ± 0.019	0.11
Stearic	37	28	3	1.17 ± 0.27	3	1.57 ± 0.25	63	0.92 ± 0.056	0.28
Myristic	37	27	3	2.21 ± 0.38	3	4.52 ± 0.93	63	0.54 ± 0.16	0.24
Lauric	37	25	3	0.156 ± 0.22	3	0.156 ± 0.18	63	0.18 ± 0.22	0.29
Lauric	37	25			6	1.39 ± 0.076	63	0.18 ± 0.22	0.29
Human Albumin									
Palmitic	23	28	3	20.19 ± 3.60	3	5.07 ± 0.84	63	0.50 ± 0.036	0.37
Palmitic	23	28	2	51.62 ± 16.0	4	9.20 ± 1.3	20	0.87 ± 0.11	0.39
Palmitic	23	28	3	20.73 ± 4.0	3	5.46 ± 0.97	20	0.91 ± 0.11	0.39
Palmitic	37	76	3	4.86 ± 0.47	3	2.77 ± 0.27	63	0.35 ± 0.13	0.32
Rabbit Albumin									
Palmitic	37	28	3	5.16 ± 0.56	3	3.64 ± 0.48	63	0.48 ± 0.072	0.24
Palmitic	37	28	2	8.68 ± 1.7	4	5.69 ± 0.60	20	1.5 ± 0.25	0.26

* Root-mean-square error is the square root of the average square difference between the computed data and the experimental data. This parameter is a measure of the average deviation of the data from the computed curve.

† Parameter uncertainty, i.e., asymptotic estimates of the standard error (see Methods section).

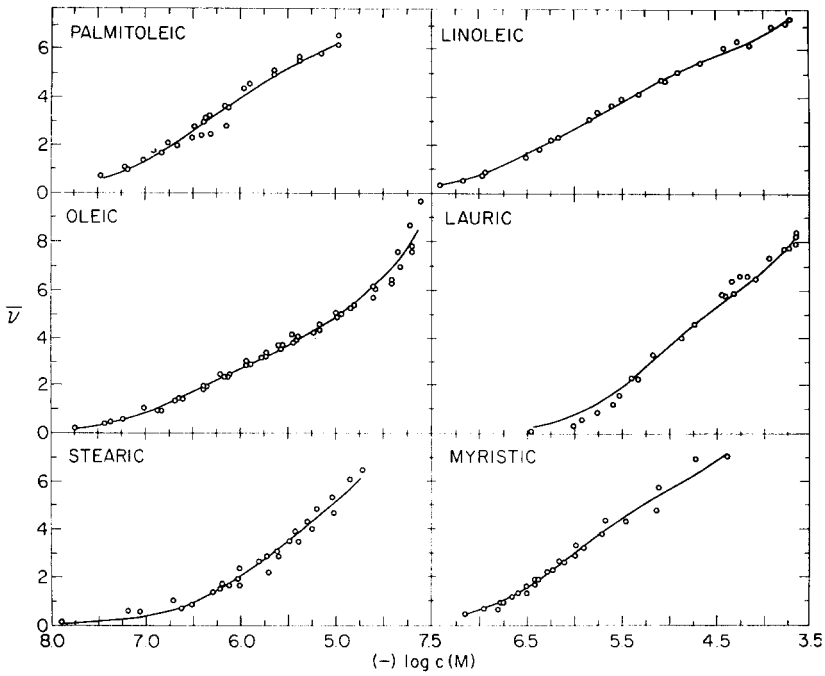

FIG. 2. Graphs for the binding of radioactive free fatty acids to crystalline bovine serum albumin at 37°C in phosphate-buffered salt solution, pH 7.4.

fit with our data. The binding curves indicate that palmitate is bound somewhat more tightly by BSA than by either HSA or RSA. To compare the affinity of the albumins under more carefully controlled conditions, we did experiments with flasks containing two compartments for aqueous solutions. In this way, two albumin solutions were exposed to the same heptane phase, and the unbound FFA concentration in both of them was identical. Under these conditions, also, palmitate was bound more tightly to BSA than to HSA (Table 4). However, the affinity of BSA was not greater than HSA for all of the fatty acids that we tested. Both oleate and laurate were bound more tightly to HSA than to BSA.

As can be seen from the representative experiments shown in Table 5, enough fatty acid was present in the heptane phase so that the maximum $\bar{\nu}$ values obtained were not limited by FFA availability. In experiment 1, the BSA concentration was 0.36 mM, the usual protein concentration that was used in the above experiments. Stearate-1-^{14}C was the ligand. Under these conditions, the total quantity of FFA in the aqueous phase changed very little when the stearate concentration in heptane exceeded 17 mM. Similar results were obtained in experiment 2, where palmitate-1-^{14}C binding was tested in a system containing 0.089 mM BSA. The total amount of FFA in the aqueous phase did not change appreciably when the palmitate concentration was raised above 2.3 mM. Likewise, a large excess of labeled fatty acid was available on Celite when maximum $\bar{\nu}$ values were attained. For example, the $\bar{\nu}$ with stearate increased from 0.6 to 5.5 when the Celite content was raised from 2 to 25 mg. When the Celite content was increased further to 100 mg, $\bar{\nu}$ increased only to 6.1. Higher $\bar{\nu}$ values were not produced by further increasing the Celite content.

Graphs of total FFA concentration [−log c (M)] in protein-free phosphate-buffered salt solution as a function of FFA concentration in heptane for palmitate, stearate, laurate, and oleate are shown in Fig. 4. As the concentration of each fatty acid in heptane increased,

TABLE 4 COMPARISON OF THE AFFINITY OF HUMAN AND BOVINE ALBUMIN FOR DIFFERENT FFA*

Experiment Number	FFA	Initial FFA Concentration in Heptane	Ratio of FFA Uptakes (Human/Bovine)†
		μeq/ml	
1	Palmitate	0.85	0.798
2	Palmitate	5.0	0.959
3	Palmitate	10.0	0.903
4	Oleate	5.0	1.02
5	Oleate	10.0	1.05
6	Laurate	10.0	1.23

* These incubations were done in a cell with two compartments. BSA (3 ml, 0.2 μmole/ml) in phosphate-buffered salt solution was put into one compartment; HSA (3 ml, 0.2 μmole/ml) was put into the other compartment. Heptane (6 ml) containing radioactive FFA was layered over the protein solutions. Both compartments containing aqueous phase were in contact at all times with the same organic phase. Incubation was done at 37°C for 24 hr with shaking.

† Two determinations of the FFA content of the BSA and HSA solutions were made. The value listed is the ratio of the FFA contained in the HSA solution to that contained in the BSA solution.

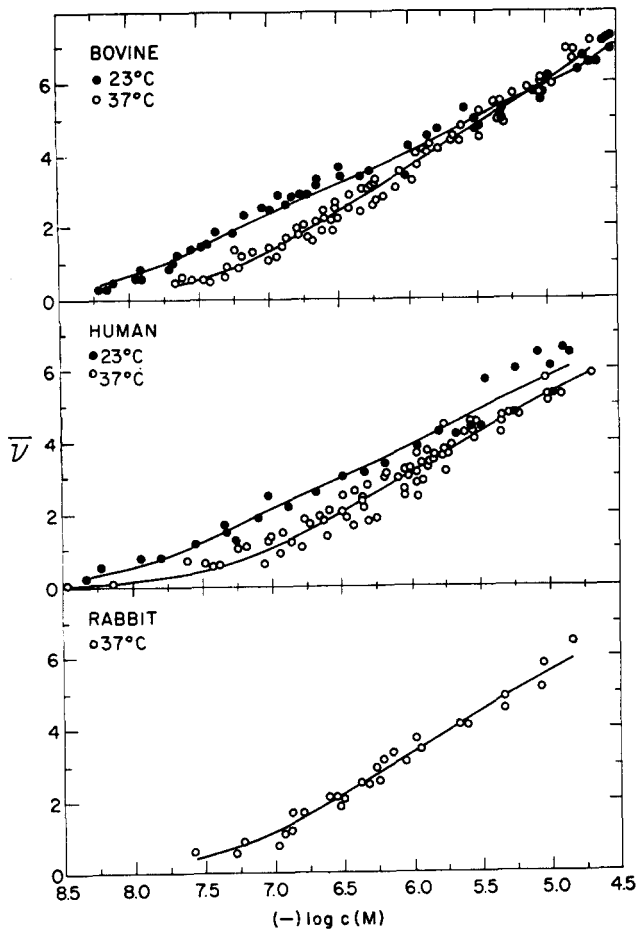

FIG. 3. Graphs for the binding of palmitic acid-1-^{14}C to crystalline bovine, human, and rabbit serum albumins at 37°C in phosphate-buffered salt solution, pH 7.4.

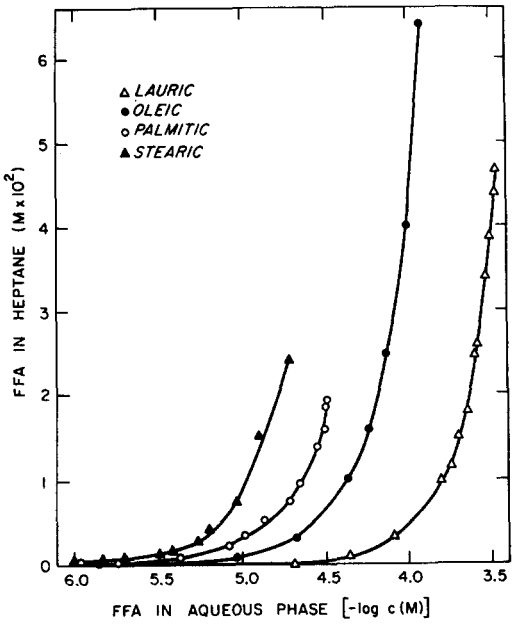

FIG. 4. Relationship between heptane and aqueous FFA concentrations in the absence of protein. Incubations were done in phosphate-buffered salt solutions, pH 7.4, at 37°C.

TABLE 5 EFFECT OF FFA CONCENTRATION IN HEPTANE ON THAT IN THE AQUEOUS PHASE AT EQUILIBRIUM

Experiment 1*		Experiment 2	
Heptane†	Aqueous‡	Heptane	Aqueous
μeq		*μeq*	
0.30	0.50	0.028	0.28
0.80	0.82	0.11	0.34
1.9	0.84	0.21	0.38
3.2	1.1	0.53	0.42
5.5	0.98	0.75	0.42
8.9	1.4	1.4	0.43
17	1.6	1.9	0.44
29	1.7	2.3	0.49
41	1.8	4.3	0.47

* Stearic acid-1-^{14}C was used in experiment 1. The BSA concentration in the aqueous phase was 3.6×10^{-4} M. Palmitic acid-1-^{14}C was used in experiment 2. The BSA concentration in the aqueous phase was 8.9×10^{-5} M. The total volume of each aqueous phase was 1 ml.

† Total fatty acid at equilibrium in the heptane phases of the flasks containing albumin. The total volume of each heptane phase was 1 ml.

‡ Total fatty acid at equilibrium in the aqueous phases containing albumin. In each case, at least 99% of the FFA present in these phases was bound to the protein.

the fatty acid concentration in the aqueous phase approached a maximum value. The highest aqueous FFA concentration that was reached varied from approximately −log 4.8 M for stearate to −log 3.5 M for laurate. Likewise, in the Celite experiments, the maximum stearate concentration attained in the absence of protein was −log 4.7 M. The data in Fig. 2 indicate that the unbound FFA concentration present at the highest $\bar{\nu}$ value obtained with each of these acids is similar to the maximum aqueous concentrations obtained in the partition experiments. Thus, it is likely that the observed maximum $\bar{\nu}$ values were limited by FFA solubility in the aqueous phase.

No differences in the palmitate–BSA binding isotherm were noted over the range of albumin concentrations used (i.e., 0.089–0.40 mM). This was expected from theoretical considerations since the parameters in the binding equation are $\bar{\nu}$ and c and not protein concentration. Almost identical data were obtained when palmitate binding was tested with five different preparations of crystalline BSA, each from a different commercial lot (Armour C 70603, C 71002, A 69805; Pentex 14; Nutritional Biochemicals 4710). Similar results for palmitate binding also were obtained with crystalline BSA that had been extracted by the method of Goodman (23).

Effects of Temperature

Palmitate was bound more tightly by both human and bovine albumin at 23°C than at 37°C (Fig. 3 and Table 3). The largest differences occurred at low values of $\bar{\nu}$. Table 6 contains thermodynamic parameters calculated

TABLE 6 THERMODYNAMIC CONSTANTS FOR THE ASSOCIATION OF PALMITATE WITH BOVINE AND HUMAN SERUM ALBUMIN

Parameter	Units	Values: Primary Sites	Values: Secondary Sites
Bovine albumin			
$\Delta F°_{37°C}$	kcal/mole	− 9.7	−8.1
$\Delta H°$	kcal/mole	−17.9	−2.2
$\Delta S°_{37°C}$	cal/deg mole	−26.5	+1.9
Human albumin			
$\Delta F°_{37°C}$	kcal/mole	− 9.5	−7.7
$\Delta H°$	kcal/mole	−17.7	−7.5
$\Delta S°_{37°C}$	cal/deg mole	−26.5	+0.7

from these data. Similar results were obtained with HSA and BSA. Negative enthalpy and entropy changes accompanied association of palmitate with the primary sites. Binding of palmitate to the secondary sites was associated with a smaller negative enthalpy change and a small positive entropy change. Because of the uncertainty associated with the binding constants derived for the tertiary sites, thermodynamic parameters for this interaction were not calculated.

Effect of Changes in the Incubation Medium

Palmitate binding to BSA in either 0.15 M KCl buffered with 0.02 M Tris–HCl, pH 7.4, or 0.1 M Tris-HCl, pH 7.4, was similar to that in phosphate-buffered salt solution. However, the strength of association in 0.01 M sodium phosphate, pH 7.4, decreased when the NaCl concentration was raised to 0.1 M or higher. Changes in pH of the incubation medium affected FFA binding to albumin as is shown in Fig. 5. The incubations at pH 6–8 were done in phosphate-buffered salt solution adjusted to the given pH with HCl or NaOH. Since the amount of added Cl^- or Na^+ was small relative to that already present in this medium, corrections for the variations in salt concentration were not made. The data obtained at pH 6.7, 6.3, and 6.0 for palmitate binding to BSA and HSA and for oleate binding to BSA lie below and to the right of the binding isotherm obtained at pH 7.4. Likewise, the data for palmitate binding to BSA at both pH 8 and 9 are displaced to the right of the binding isotherm for pH 7.4 at values of $\bar{\nu}$ up to 4. The data obtained at pH 10 are displaced even further to the right at values of $\bar{\nu}$ up to 5. Incubations at pH 9 and 10 were done in 0.1 M Tris–HCl buffer.

Fatty Acid Structure and Binding

Changes in the structure of the FFA chain (either length or degree of unsaturation) produced small differences in the strength of association with BSA (Table 3). In addition, binding decreased greatly when the carboxyl group was modified or removed. The highest value of $\bar{\nu}$ that was observed with hexadecane was 2.0; that with

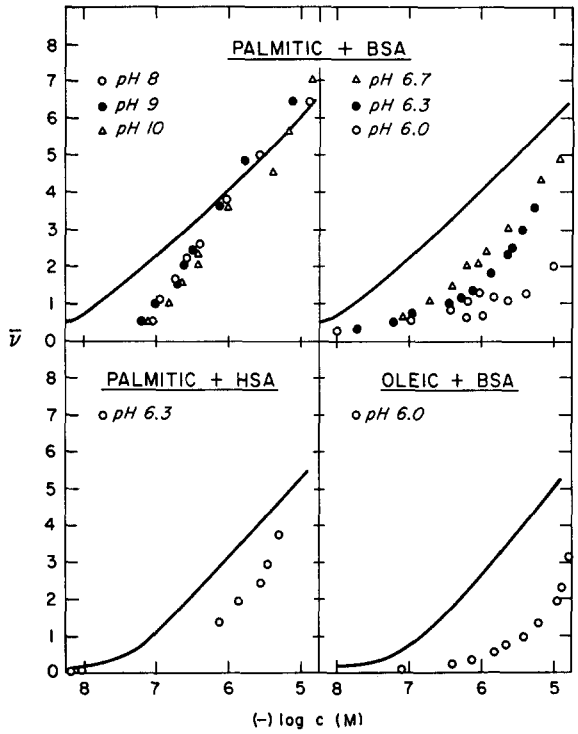

FIG. 5. Effect of pH on the binding isotherms of radioactive FFA to crystalline albumin at 37°C. The incubations at pH 6–8 were done in phosphate-buffered salt solution, those at pH 9 and 10 were done on 0.1 M Tris–HCl. The solid curves are the binding isotherms for pH 7.4, calculated from the data of Table 3.

methyl palmitate was 2.4. These values were obtained from Celite incubations. The k' for binding of hexadecane to BSA was 3.3×10^4 M^{-1}, and that for binding of methyl palmitate was 2.9×10^4 M^{-1}. Cetyl alcohol was also bound poorly by BSA. The maximum $\bar{\nu}$ value obtained in the Celite system was 1.2. The k' for binding of cetyl alcohol in the Celite system was 2.5×10^5 and in the heptane system, 2.0×10^5 M^{-1}.

Protein Structure and Binding

Ribonuclease, fibrinogen, α-amylase, trypsin, α-chymotrypsin, lysozyme, streptococcal protease, and subtilisin did not bind palmitate, i.e., they took up less than 2% of the amount that was bound by the same quantity (weight) of BSA. β-Lactoglobulin took up a maximum of about 20% as much palmitate as BSA. These measurements were made in phosphate-buffered salt solution, pH 7.4.

The binding of palmitate to preparations of BSA that were modified by physical and chemical means is illustrated in Fig. 6. Binding was depressed only slightly as compared to native BSA (curve *A*) when the protein was incubated with *O*-methylisourea so that more than 98% of the free amino groups were removed (curve *B*). A more profound decrease in binding occurred (curve *C*) when BSA was acetylated at pH 5 (87% of the free

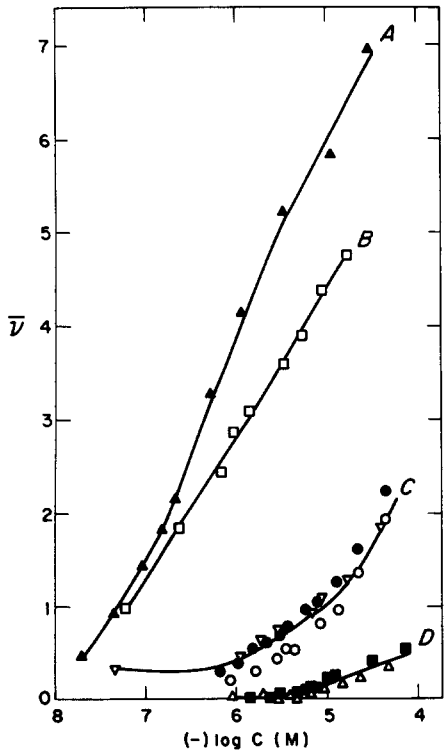

FIG. 6. Binding of palmitic acid-1-^{14}C by modified bovine serum albumins. Curve *A*, native BSA (solid triangles); curve *B*, guanidated BSA (open squares); curve *C*, acetylated (87%) BSA (solid circles), formaldehyde-treated BSA (open circles), and heat-treated BSA (open inverted triangles); curve *D*, BSA in 6 M urea (solid squares) and acetylated (98%) BSA (open triangles).

amino groups react under these conditions), heated at 75°C for 3 min, or treated with formaldehyde at pH 11. All of the free amino groups were intact in the formaldehyde-treated protein. Binding was almost totally abolished (curve *D*) when the incubation was done in the presence of 6 M urea or when the protein was acetylated at pH 8 (when 99% of the free amino groups react). Ultraviolet fluorescence spectra of these chemically modified proteins revealed shifts in the wavelength of maximum emission as well as differences in quantum yield which suggest that these procedures may have produced structural alterations in BSA.

DISCUSSION

Our data indicate that BSA contains six high-energy binding sites for long-chain FFA and a large number of weaker binding sites. The high-energy binding sites were separated into two distinct classes, each containing three sites. This model is compatible with results from studies with short-chain fatty acids (24), anionic dyes (25, 26), and detergents (27, 28) which indicate that BSA has a large capacity to bind organic ligands. Use of a model containing large numbers of binding sites can be reconciled with the experimental observations that a maximum of 6–13 moles of FFA can be bound per mole of BSA. Low maximum $\bar{\nu}$ values also were observed using a spectrophotometric assay (29) and when BSA was exposed to glass slides coated with monolayers of palmitate (30). It is likely that the maximum $\bar{\nu}$ that can be achieved experimentally is limited by the solubility of long-chain FFA in aqueous solution (i.e., unbound FFA concentration) and not by the number of BSA binding sites. If the aqueous concentration of long-chain FFA could be raised in these systems to the levels that can be attained with other, more water-soluble ligands, it is probable that much higher $\bar{\nu}$ values could have been reached. The highest aqueous concentration that could be attained was different for each acid, accounting in part for the fact that the maximum experimental $\bar{\nu}$ values vary from 6.5 for stearate to 13.5 for oleate. The actual number of weak (tertiary) binding sites could not be determined with any accuracy. Hence, the values given for k'_3 also are only gross approximations. Use of a large value for the number of tertiary sites is supported by studies with anionic detergents which demonstrate experimentally that BSA has a large binding capacity for long-chain organic ligands (28, 31). In addition, both Goodman (4) and Arvidsson (5) have presented evidence that HSA possesses a large capacity to bind FFA (5).

The model we have constructed assumes that each of the three classes of BSA sites can compete independently for FFA. However, the possibility that many or all of the weak sites are formed as a result of an alteration in BSA conformation caused by FFA binding to the high-energy sites (26, 32) cannot be excluded.

When laurate served as the ligand, the six high-energy binding sites could not be separated into two classes. Hence, the laurate data were analyzed in terms of only high- and low-energy binding sites. This two-class model is similar to the one derived for laurate binidng by Reynolds, Herbert, and Steinhardt (33) and to the model for binding of short-chain FFA to BSA derived by Teresi and Luck (24). It is likely that laurate binds to the same high-energy sites as the longer-chain FFA, for it induces identical changes in the ultraviolet fluorescence spectrum of BSA as do the longer-chain FFA at values of $\bar{\nu}$ between 1 and 6 (34).

Association of FFA with albumin is thought to involve electrostatic attraction of the FFA carboxyl group to protein cationic sites together with hydrophobic interactions between the FFA hydrocarbon tail and nonpolar side chains of the protein (4, 35–38). Our results with the FFA analogues and with chemically and physically modified albumins are compatible with this interpretation. The importance of hydrophobic interactions in FFA binding is stressed by the fact that negative enthalpy *and* entropy changes accompanied palmi-

tate binding to the primary class of sites. This thermodynamic pattern is typical of hydrophobic bonding between protein and ligand (39). The observed net negative entropy change indicates that the magnitude of hydrophobic interaction at these sites is large enough to exceed the disruptive effect caused by the accompanying electrostatic interaction. A positive entropy change was associated with binding to the secondary sites, which suggests that hydrophobic interactions make a smaller relative contribution to the energy of binding at these sites.

When the pH of the medium was lowered from pH 7.4 to 6.0, the *total* unbound FFA concentration at a given $\bar{\nu}$ increased. Similar results have been noted for 1-octanol binding to BSA (31). The most likely explanation is that the affinity of BSA for these organic ligands is dependent upon pH. However, the possibility that these phenomena result from a pH effect on the organic ligand, such as increased association in aqueous solution, must be considered. Mukerjee (40), using Goodman's data (19), has demonstrated that FFA anions associate in aqueous media. We have preliminary evidence that the amount of FFA association increases as the medium pH is lowered.[2] If BSA binds associated forms of FFA poorly relative to the FFA anion monomer, then *total* unbound FFA concentration at a given $\bar{\nu}$ would be expected to increase as the pH was lowered even though the affinity of BSA for ligand did not change. Since pH-dependency of binding also occurred with 1-octanol, a nonionizable ligand, the possibility of association of unionized organic molecules in aqueous solution also must be considered.

In contrast with the findings of others (19,40), we were able to analyze our heptane–water partition data for FFA *at pH 7.4* without assuming the existence of fatty acid association in the aqueous phase. The FFA binding data were calculated with these partition results. However, the FFA partition data at pH 7.4 could be fitted equally well to a model that contained an aqueous dimerization term.[2] Certain physicochemical inconsistencies associated with the values for the FFA dimerization constants in heptane that were derived by the former analysis are corrected by assuming the presence of FFA dimerization in the aqueous phase.[2] Therefore, it is possible that small errors have been introduced into the calculation of the FFA association constants at pH 7.4 because total unbound FFA concentration was used rather than the actual concentration of the predominant species that binds: the FFA anion. Since aqueous FFA association does not become appreciable until relatively high FFA concentrations are reached (40), the errors, if they actually occur, should be confined almost entirely to the secondary and tertiary binding constants.

[2] R. B. Simpson and A. A. Spector, unpublished observations.

We thank Dr. Raymond F. Chen for assistance with the spectrofluorimetric assays and the extraction of endogenous fatty acids from serum albumin; Dr. Robert I. Levy for performing the electrophoretic determinations; Dr. DeWitt S. Goodman and Dr. Richard B. Simpson for advice during the preparations of this manuscript; and Dr. Daniel Steinberg for many helpful discussions during the course of this study.

Manuscript received 30 January 1968; accepted 23 September 1968.

References

1. Spector, A. A., and K. M. John. 1967. *Circulation.* **36** (Suppl. II): 38.
2. Dole, V. P. 1956. *J. Clin. Invest.* **35:** 150.
3. Gordon, R. S., Jr., and A. Cherkes. 1956. *J. Clin. Invest.* **35:** 206.
4. Goodman, DeW. S. 1958. *J. Amer. Chem. Soc.* **80:** 3892.
5. Arvidsson, E. O. 1965. Small molecule–protein interactions. Thesis. University of Lund, Sweden.
6. Foster, J. F. 1960. *In* The Plasma Proteins. F. W. Putnam, editor. Academic Press, Inc., New York. **1:** 177–233.
7. Morrison, W. R., and L. M. Smith. 1964. *J. Lipid Res.* **5:** 600.
8. Spector, A. A., D. Steinberg, and A. Tanaka. 1965. *J. Biol. Chem.* **240:** 1032.
9. Spector, A. A., and D. Steinberg. 1966. *J. Lipid Res.* **7:** 649.
10. Chen, R. F. 1967. *J. Biol. Chem.* **242:** 173.
11. Levy, R. I., and D. S. Fredrickson. 1965. *J. Clin. Invest.* **44:** 426.
12. Fraenkel-Conrat, H., R. S. Bean, and H. Lineweaver. 1949. *J. Biol. Chem.* **177:** 385.
13. Tabachnick, M. 1964. *J. Biol. Chem.* **239:** 1242.
14. Teresi, J. D. 1950. *J. Amer. Chem. Soc.* **72:** 3972.
15. Moore, S., and W. H. Stein. 1948. *J. Biol. Chem.* **176:** 367.
16. Gornall, A. G., C. J. Bardewill, and M. M. David. 1949. *J. Biol. Chem.* **177:** 751.
17. Baxter, J. H. 1964. *Arch. Biochem. Biophys.* **108:** 375.
18. Steiner, R. F., and H. Edelhoch. 1963. *Biochim. Biophys. Acta.* **66:** 341.
19. Goodman, DeW. S. 1958. *J. Amer. Chem. Soc.* **80:** 3887.
20. Scatchard, G. 1949. *Ann. N.Y. Acad. Sci.* **51:** 660.
21. Fletcher, J. E., and A. A. Spector, 1968. *Computers Biomed. Res.* In press.
22. Avigan, J. 1959. *J. Biol. Chem.* **234:** 787.
23. Goodman, DeW. S. 1957. *Science.* **125:** 1296.
24. Teresi, J. D., and J. M. Luck. 1952. *J. Biol. Chem.* **194:** 823.
25. Klotz, I. M., F. M. Walker, and R. B. Pivan. 1946. *J. Amer. Chem. Soc.* **68:** 1486.
26. Karush, F. 1952. *J. Phys. Chem.* **56:** 70.
27. Markus, G., and F. Karush. 1957. *J. Amer. Chem. Soc.* **79:** 3264.
28. Reynolds, J. A., S. Herbert, H. Polet, and J. Steinhardt. 1967. *Biochemistry.* **6:** 937.
29. Campbell, J., A. D. Martucci, and G. R. Green. 1964. *Biochem. J.* **93:** 183.
30. Kessler, J. I., M. Demeny, and H. Sobotka. 1967. *J. Lipid Res.* **8:** 185.
31. Ray, A., J. A. Reynolds, H. Polet, and J. Steinhardt. 1966. *Biochemistry.* **5:** 2606.
32. Klotz, I. M., and J. Ayers. 1953. *Discussions Faraday Soc.* **13:** 189.

33. Reynolds, J., S. Herbert, and J. Steinhardt. 1967. *Biochemistry*. **7:** 1357.
34. Spector, A. A., and K. M. John. 1968. *Arch. Biochem. Biophys*. **127:** 65.
35. Boyer, P. D., G. A. Ballou, and J. M. Luck. 1946. *J. Biol. Chem*. **162:** 199.
36. Boyer, P. D., F. G. Lum, G. A. Ballou, J. M. Luck, and R. G. Rice. 1946. *J. Biol. Chem*. **162:** 181.
37. Klotz, I. M., and F. M. Walker. 1947. *J. Amer. Chem. Soc*. **69:** 1609.
38. Teresi, J. D. 1950. *J. Amer. Chem. Soc*. **72:** 3972.
39. Crothers, D. M., and D. I. Ratner. 1968. *Biochemistry*. **7:** 1823.
40. Mukerjee, P. 1965. *J. Phys. Chem*. **69:** 2821.