TABLE II

REPORTED VALUES OF THE DIMERIZATION (ASSOCIATION) CONSTANTS OF FATTY ACIDS IN ANHYDROUS ORGANIC SOLVENTS, 30°[a]

Acid	Solvent	k_d[b]
Formic	Benzene	1.3×10^2
Acetic	Benzene	3.7×10^2
Propionic	Benzene	3.8×10^2
Butyric	Benzene	4.3×10^2
Stearic	Benzene	5.2×10^2
Formic	Heptane	2×10^4
Acetic	Heptane	4×10^4

[a] Data of Pohl, Hobbs and Gross[9,10] and Maryott, Hobbs and Gross.[11] [b] Calculated from their reported dissociation constants, after correcting their constants (given in units of moles/molar volume of solvent) to units of moles/liter.

should also be noted that the estimated values of k_d for formic and acetic acids in anhydrous heptane (Table II) are greater than the values obtained in this study (Table I).[32] Part of this difference may reside in the fact that in the present studies the solvent is heptane saturated with water, compared to anhydrous heptane (*vide supra*). Also relevant is the fact that the constants reported by Pohl, Hobbs and Gross for heptane are admittedly only estimates, since they were unable to make sufficiently precise measurements in solutions of sufficiently low concentration in heptane.

In conclusion, the magnitude of the values of k_d observed in these studies deserves re-emphasis. In heptane solution dimerization is significant at concentrations well below 10^{-4} molar, and the dimer is the stable and predominant form over a wide range of concentration. The studies of Pauling and Brockway[33] and others have shown that the configuration of such dimers involves a symmetrical coplanar structure of the two carboxyl groups, connected by two hydrogen bonds. The magnitude of k_d herein observed is a testimony to the great stability of this structure.

Acknowledgment.—I would like to thank Dr. Robert S. Gordon, Jr., for a great deal of valuable advice and assistance.

(32) Reasoning from the values for k_d for benzene given in Table II, one would expect k_d in heptane for the acids longer than acetic (*e.g.*, those in Table I) to be greater than k_d for acetic and formic acids in heptane.

(33) L. Pauling and L. O. Brockway, *Proc. Natl. Acad. Sci.*, **20**, 336 (1934).

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The Interaction of Human Serum Albumin with Long-chain Fatty Acid Anions

BY DEWITT S. GOODMAN

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The interaction of human serum albumin with six long-chain fatty acid anions has been studied by the technique of partition analysis, in which varying quantities of each fatty acid were equilibrated between two phases. The phases employed were *n*-heptane and an aqueous solution of serum albumin in phosphate buffer, pH 7.45, ionic strength 0.16, at 23°. The concentration of unbound fatty acid in each aqueous phase was determined from the concentration in heptane, using the results of identical distribution experiments performed in the absence of albumin. The fatty acids studied included lauric, myristic, palmitic, stearic, oleic and linoleic acids. The human serum albumin was a special preparation with a fatty acid content of 0.1 mole/mole or less. The data obtained have been analyzed in terms of three classes of binding sites and the apparent association constants determined. The first class consists of 2 sites, the second of 5, and the third of a larger number, arbitrarily taken to be 20. The values of k_1' for the different fatty acids are: laurate 1.6×10^6; myristate 4.0×10^6; palmitate 6.0×10^7; stearate 8.0×10^7; oleate 1.1×10^8; linoleate 1.3×10^7. The relationship between the apparent and the intrinsic association constants is discussed in detail. The possible structural specificity of the different classes of binding sites is also discussed, as are some of the implications for metabolic studies.

It has long been known that serum albumin interacts strongly with fatty acid anions. In 1941 Kendall found that crystalline albumin was always associated with a small amount of free fatty acid that could not be removed by repeated crystallization.[1] Since then the nature and effects of this interaction have been the subjects of numerous investigations. Thus, fatty acid anions have been shown to stabilize serum albumin against denaturation by urea, guanidine and heat[2,3] and to compete effectively with organic dyes for binding sites on the albumin molecule.[4,5] Electrophoretic studies have demonstrated an increase in the mobility of serum albumin in the presence of fatty acid salts.[6,7] Furthermore, Davis and Dubos observed that serum albumin acts as a protective growth factor for tubercle bacilli by binding traces of fatty acids in the medium and that serum albumin could protect sheep erythrocytes against hemolysis by oleic acid.[8] Most of the available studies have, however, been indirect, qualitative or at best semi-quantitative. An outstanding exception to this statement is the study by Teresi and Luck, in which the binding of several short-chain fatty acid anions (8C and less in length) to bovine serum albumin was quantitatively studied by the method of equilibrium dialysis.[9] This investigation suffered, however,

(1) F. E. Kendall, *J. Biol. Chem.*, **138**, 97 (1941).

(2) P. D. Boyer, F. G. Lum, G. A. Ballou, J. M. Luck and R. G. Rice, *ibid.*, **162**, 181 (1946).

(3) P. D. Boyer, G. A. Ballou and J. M. Luck, *ibid.*, **162**, 199 (1946).

(4) G. E. Cogin and B. D. Davis, THIS JOURNAL, **73**, 3135 (1951).

(5) U. Westphal, J. P. Stets and S. G. Priest, *Arch. Biochem. Biophys.*, **43**, 463 (1953).

(6) G. A. Ballou, P. D. Boyer and J. M. Luck, *J. Biol. Chem.*, **159**, 111 (1945).

(7) R. S. Gordon, Jr., *J. Clin. Invs.*, **34**, 477 (1955).

(8) B. D. Davis and R. J. Dubos, *J. Exp. Med.*, **86**, 215 (1947).

(9) J. D. Teresi and J. M. Luck, *J. Biol. Chem.*, **194**, 823 (1952).

from the limitation that the initial content of fatty acid of the bovine albumin used in the experiments was not known.[10] No quantitative data are available concerning the binding of long-chain fatty acid anions to serum albumin.

More recently, there has been increasing interest among biochemists and physiologists in the interaction of serum albumin with long-chain fatty acid anions because of the demonstration of the importance of the small quantity of unesterified fatty acid present in the blood plasma of mammals.[11-13] This fatty acid exists in plasma as a complex with serum albumin. Furthermore, albumin has been shown to play an essential role in the lipemia clearing reaction, both *in vivo* and *in vitro*, by binding fatty acids released during lipolysis.[14-16]

In the present study the interaction of human serum albumin with six long-chain fatty acid anions has been studied quantitatively by the method of partition analysis (*vide infra*).[17] The use of C^{14}-labeled fatty acids has permitted measurements at extremely low concentrations. The data obtained have been analyzed in terms of three classes of binding sites, and the apparent association constants determined.

Experimental

Materials.—Both the non-radioactive and the C^{14}-labeled fatty acids used in this study (lauric, myristic, palmitic, stearic, oleic and linoleic acids) were identical with those described in the preceding paper.[18] The solutions of the sodium salts of these fatty acids were also identical with those therein described,[18] as were the inorganic and organic reagents. The same phosphate buffer was used in both studies[18]; it was prepared as a stock concentrated solution, a 1 in 10 dilution of which resulted in a solution of *p*H 7.45 and ionic strength 0.160. The human serum albumin used in these experiments was identical with a previously described preparation.[19] This albumin was a sample of fraction V, the long-chain fatty acid content of which had been reduced to 0.1 mole/mole or less by extracting the lyophilized protein with 5% glacial acetic acid in isoöctane. The extracted albumin was indistinguishable from the untreated albumin as regards its ultracentrifugal, electrophoretic and immunologic properties, its optical rotation and its binding of methyl orange anions.[19] Albumin solutions were freshly prepared for each study by dissolving some of the lyophilized protein (stored at −10°) in distilled water. The concentration of each solution was determined by comparing its optical density at 279 mμ as read in a Beckman quartz spectrophotometer to the optical density of a standard albumin solution whose concentration was known.[20] The weight concentration was converted to molar concentration by taking 69000 as the molecular weight.[21,22] In each study the albumin solution so prepared was of concentration between 5 and 6×10^{-4} mole/liter.

Equilibration of the Two Phases.—The experimental technique employed consisted in the equilibration of varying quantities of the fatty acid being studied between two phases, *n*-heptane and an aqueous solution of serum albumin in phosphate buffer. In each study equilibration was conducted in a series of 60-cc. separatory funnels, the stems of which had been cut off prior to use. To each funnel were added 2 cc. of the concentrated phosphate buffer, 2 cc. of the serum albumin solution, 1 cc. of the C^{14}-labeled fatty acid salt solution, 0 to 15 cc. of one or another of the unlabeled fatty acid salt solutions and enough distilled water to make a total volume of 20 cc. The final concentration of serum albumin in these studies was hence between 5 and 6×10^{-5} mole/liter in each case. The total quantity of fatty acid in each series of funnels ranged over more than two orders of magnitude (from mole ratios of 0.1 to 0.2 to mole ratios of 20 to 50, depending on the fatty acid's solubility). In the studies with myristate, palmitate, stearate and oleate, the fatty acids were insoluble in the funnels containing the largest amounts of material, before the addition of heptane.[24] To each funnel 2 cc. of *n*-heptane was then added, and the funnels equilibrated by being gently tilted back and forth, at a rate of 15 to 20 times per minute, for 50 to 60 hours in an air-conditioned laboratory at 23 ± 1°. Forty-eight hours were found sufficient for attainment of equilibrium. After equilibration the funnels were centrifuged for 15 minutes at 500 r.p.m. and each aqueous phase drawn off and collected. Centrifugation was then repeated to ensure that the heptane phases were free of tiny droplets of aqueous phase.

Analyses after Equilibration.—The concentration of radioactivity in each upper (heptane) phase was measured with a Packard Tri-Carb Liquid Scintillation Spectrometer, in the manner described in the preceding paper.[18] The concentration of radioactivity in the aqueous phases was determined by plating 0.20 cc. of each solution on a stainless steel planchet of area 1.43 sq. cm., allowing the planchets to dry at room temperature and counting them in a Robinson gas flow counter.[25] Duplicate measurements were made on each lower phase and agreed within 2% of each other. Sufficient counts were recorded so that the standard error of each measurement was 2% or less.[26] The concentration of radioactivity (c.p.m./cc.) so determined for each lower phase was then converted to equivalent c.p.m./cc. in the Tri-Carb Scintillation Spectrometer (under the same conditions as obtained for the measurements of the upper phases) by use of a separately determined correction factor. This correction factor was determined by counting 0.20 cc. of a solution of palmitic acid-1-C^{14} in heptane in both the scintillation spectrometer and the gas-flow counter. Measurements were made in quadruplicate, and those with the gas-flow counter were made only after 0.20 cc. of a solution of serum albumin in phosphate buffer (of concentrations identical with those in these studies) had been added to each of the planchets. The desired correction factor was thus the ratio of the c.p.m. observed with the two instruments. This factor corrected for both the difference in efficiency of the two instruments and the "self-absorption" of albumin and phosphate on the planchets.

Preliminary Calculations.—After determining the number of counts per minute (c.p.m.) per cc. for each heptane and aqueous phase, the total number of c.p.m. in each system (*i.e.*, each sep. funnel) was calculated. The recovery of c.p.m. was within 5% of the expected recovery for each two phase system. The total concentration of fatty acid molecules (in all forms) in each heptane and aqueous phase was calculated from these values, together with the known volumes and the known amounts of fatty acid in each sys-

(10) Crystalline bovine albumin has been found, in this Laboratory, to usually contain about 2 moles of long-chain fatty acid per mole of albumin (unpublished observations).

(11) R. S. Gordon, Jr., and A. Cherkes, *J. Clin. Inv.*, **35**, 206 (1956).

(12) R. S. Gordon, Jr., *ibid.*, **36**, 810 (1957).

(13) V. P. Dole, *ibid.*, **35**, 150 (1956).

(14) R. S. Gordon, Jr., E. Boyle, R. K. Brown, A. Cherkes and C. B. Anfinsen, Jr., *Proc. Soc. Exp. Med. Biol.*, **84**, 168 (1953).

(15) D. S. Robinson and J. E. French, *Quart. J. Exp. Physiol.*, **38**, 233 (1953).

(16) R. H. Rosenman and M. Friedman, *J. Clin. Inv.*, **36**, 700 (1957).

(17) The simpler and more direct method of equilibrium dialysis could not be used for the long-chain saturated fatty acid anions, because these fail to pass through dialysis membranes which retain albumin. The reason for this is not known.

(18) D. S. Goodman, THIS JOURNAL, **80**, 3887 (1958).

(19) D. S. Goodman, *Science*, **125**, 1296 (1957).

(20) The concentration of the standard solution was determined by Kjeldahl analysis for nitrogen, using the factor of 6.25 to convert weight of nitrogen to weight of protein.

(21) G. Scatchard, A. C. Batchelder and A. Brown, THIS JOURNAL, **68**, 2320 (1946).

(22) It is recognized that recent studies indicate the molecular weight of serum albumin to be close to 66000.[23] 69000 has been used here, however, to conform with usage in other binding studies with albumin, and because of the heterogeneity of this fraction V preparation as compared to crystalline mercaptalbumin.

(23) B. W. Low, THIS JOURNAL, **74**, 4830 (1952).

(24) Turbidity appeared when one added more than about 5 moles of stearate, 8 moles of palmitate, 13 moles of myristate or 22 moles of oleate per mole of serum albumin to this system.

(25) C. V. Robinson, *Science*, **112**, 198 (1950).

(26) D. Steinberg and S. Udenfriend, Article 20 in "Methods in Enzymology," Vol. IV, Academic Press, Inc., New York, N. Y., 1957.

tem. From the concentration in the heptane phase the free (unbound) fatty acid concentration in the aqueous phase was determined by dividing the concentration in heptane by the partition ratio appropriate for that precise concentration. The partition ratio is defined as the total concentration of fatty acid in heptane divided by the total concentration in phosphate buffer, at equilibrium, in the absence of albumin, for the conditions of this study (*p*H 7.45, ionic strength 0.160, 23°). The rationale for this procedure is that two solutions, one with and one without albumin, which are in equilibrium with the same concentration of fatty acid in heptane are in equilibrium with each other. The complexity of this procedure derives from the fact that the partition ratio as defined above varies with fatty acid concentration. This has been described and discussed in detail in the preceding paper.[18] It was hence necessary to measure the partition ratio over a wide range of fatty acid concentration for each fatty acid studied. The results of these measurements are presented graphically in the preceding paper.[18] Using these results (Figs. 3, 4, 5 and 6 of the preceding paper), it was hence possible to obtain the proper partition ratio for any given concentration of fatty acid in heptane.

The concentration of bound fatty acid in each aqueous phase was next calculated as the difference between the total concentration and the unbound concentration in the aqueous phase. Finally, the average number of fatty acid ions bound per albumin molecule, $\bar{\nu}$, was determined by dividing the molar concentration of bound fatty acid by the molar concentration of albumin.

Validity of the Method.—Before presenting the results, the possible objections to the use of a two-phase system for studies of this nature should be considered. Two major objections may be made: (a) the two-phase equilibration may result in some denaturation of albumin, and (b) the heptane may interfere with fatty acid binding by itself binding to albumin. As regards the first objection, there was no visible denaturation at the interface in any of the experiments, and the albumin after equilibration was indistinguishable ultracentrifugally from albumin before equilibration. It is hence felt that denaturation, if it occurs at all, is minimal in this system. The second objection derives from the observation of Karush[27] that the binding of an anionic azo dye to bovine albumin was decreased if the system was saturated with toluene, presumably because of toluene binding to albumin. The solubility of heptane in water (5×10^{-4} molar at 18°)[28] is, however, approximately one-tenth the solubility of toluene in water. This fact, together with the extreme tightness of the fatty acid binding (see Results), makes it quite unlikely that heptane binding to albumin could significantly interfere with fatty acid binding. In this regard it should be noted that Schellman, *et al.*, obtained the same results for the binding of testosterone to bovine serum albumin by the methods of equilibrium dialysis and of partition analysis using heptane.[29]

Results and Discussion

The results of these studies are presented graphically in Fig. 1 through 6. In Fig. 1 through 3, $\bar{\nu}$ is plotted against the negative logarithm of c, where c is the concentration of unbound (free) fatty acid in the aqueous phase. This is the familiar form of a titration curve. In Figs. 4 through 6 $\bar{\nu}/c$ is plotted against $\bar{\nu}$. The curves drawn in these figures are plots of equation 1 discussed below.

Analysis in Terms of Apparent Classes and Association Constants.—As indicated by Scatchard[30-32] the binding of an anion A to several classes of sites on a protein molecule may be formulated as

$$\nu_A = \sum_i \bar{\nu}_{Ai} = \sum_i \frac{n_i k'_{Ai} c_A}{1 + k'_{Ai} c_A} \qquad (1)$$

in which k'_{Ai} is the apparent association constant for each of the n_i sites in class i under any particular set of conditions, and c_A is the free (unbound) concentration of A in equilibrium with the protein.[33] The apparent association constant, k'_{Ai}, is related to the intrinsic association constant, k^0_{Ai}, by a number of factors which correct for the effects of electrostatic interactions, competition with other ions and the hydrogen ion equilibrium of the binding sites. These factors will be discussed in detail below.

The data obtained in these studies and plotted in Figs. 1 through 6 were analyzed in terms of eq. 1, so as to obtain the values of n_i and k'_{Ai} for each of the six fatty acids studied, under the conditions here employed (phosphate buffer *p*H 7.45, ionic strength 0.160, 23°). Inspection of eq. 1 reveals that if there is only one class of sites a plot of $\bar{\nu}/c$ *vs.* $\bar{\nu}$ should be a straight line, whereas if there are more than one class of sites such a plot should be a curve which is concave upwards. Figures 4 through 6 indicate that in these studies the latter is indeed the case, *i.e.*, the data must be analyzed in terms of more than one class of sites. Further analysis of the data (*cf.* Scatchard, *et al.*),[32] revealed that in all cases the data could be best fitted by assumption of at least three classes of binding sites, and, in fact, the results with four of the fatty acids (laurate, myristate, oleate and linoleate) could not be fitted with less than three classes.

The data for each of the fatty acids were then separately analyzed in terms of 3 classes of binding sites. In every case the value of n_1 was found to be 2. The values of n_2 were less precisely determinable than those of n_1, because of the greater experimental error at higher fatty acid concentrations. The best values of n_2 were found to be 6 for laurate and myristate, 4 for palmitate and stearate and 5 for oleate and linoleate with an estimated error of ± 1 in each case. The values of n_3 could not be determined with any accuracy, except for the fact that n_3 appeared to be larger than n_2. The identity of the values of n_1, and the closeness of the values of n_2, for the six fatty acids studied then permitted the assumption that all six fatty acids bind to the same classes of sites on the albumin molecule. The best single set of values for n_1, n_2 and n_3 which would fit all the data was then selected. These values are $n_1 = 2$, $n_2 = 5 \pm 1$ and $n_3 = 20$ (with a large error which cannot be accurately estimated). The value of $n_3 = 20$ was arbitrarily selected so that the sum of n_1, n_2 and n_3 would be identical with the sum of n_1, n_2 and n_3 given by Scatchard for the binding of simple inorganic ions to bovine serum mercaptalbumin.[32] Using these values of n, the values of k_1', k_2' and k_3' which would best fit the data were then determined for each of the six fatty acids. These values are listed in Table I. The curves drawn in Figs. 1 through 6 are all plots of equation 1 for three classes of binding sites, using the values of n listed

(27) F. Karush, THIS JOURNAL, **72**, 2705 (1950).

(28) "The Handbook of Chemistry," 9th Ed., N. A. Lange, ed., Handbook Publishers, Sandusky, Ohio, 1956.

(29) J. A. Schellman, R. Lumry and L. T. Samuels, THIS JOURNAL, **76**, 2808 (1954).

(30) G. Scatchard, *Ann. N. Y. Acad. Sci.*, **51**, 660 (1949).

(31) G. Scatchard, I. H. Scheinberg and S. H. Armstrong, Jr., THIS JOURNAL, **72**, 535, 540 (1950).

(32) G. Scatchard, J. B. Coleman and A. L. Shen, *ibid.*, **79**, 17 (1957).

(33) Strictly speaking, the activity of A should be used instead of the concentration. Fatty acid concentrations were used in the calculations here presented. This introduced a small systematic error in all the results, of equal magnitude in all the studies.

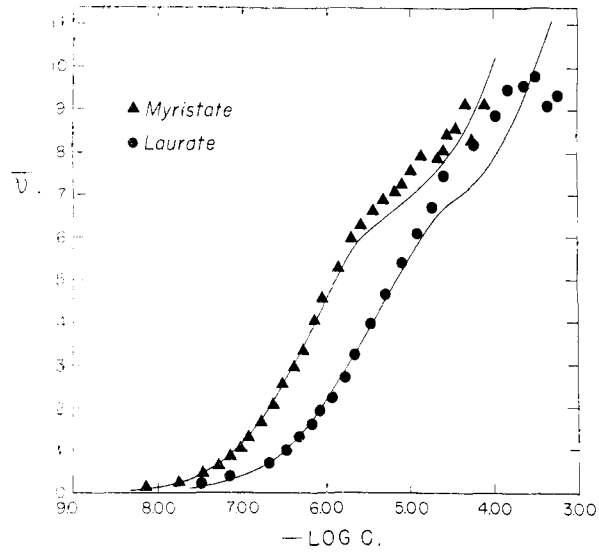

Fig. 1.

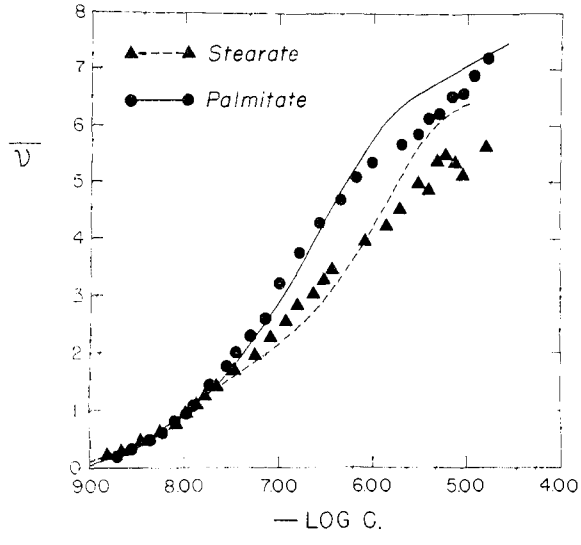

Fig. 2.

above and the values of k' listed in Table I. Inspection of the figures indicates that these values of n and k' fit the data satisfactorily in every case.[34]

The relative reliability of the values of k_1', k_2' and k_3' is similar to the relative reliability of the values of n_1, n_2 and n_3. Thus, the values for k_1' are most accurate, because the experimental error was smallest for those points with the lowest concentrations of fatty acid. The values of k_2' are much less accurate, and the error in these values is probably greater than 50% in one or two cases (particularly for stearate). This is due both to the fact that the experimental error increased considerably as fatty acid concentration increased and to the possibility that association between fatty acid ions may have occurred at the higher concentrations studied. Such association certainly occurred in the cases of stearate and palmitate, for the aqueous phases

(34) The fit is best, in all cases, for low values of $\bar{\nu}$. As discussed above, the fit for higher values of $\bar{\nu}$ would be somewhat better if n_3 = 6 was used for myristate and laurate, and n_3 = 4 for stearate and palmitate.

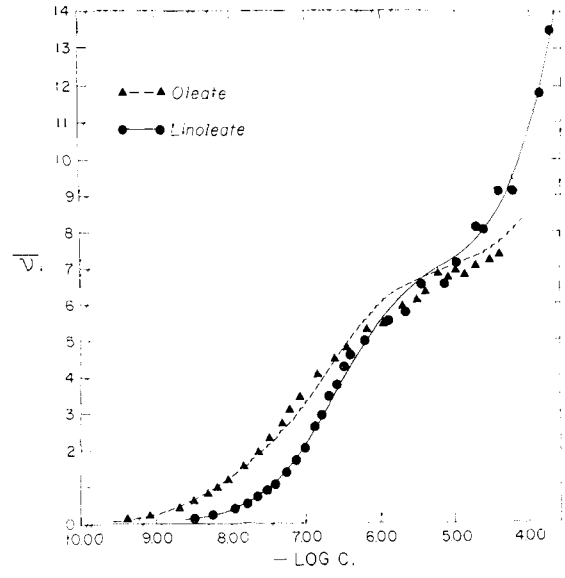

Fig. 3.

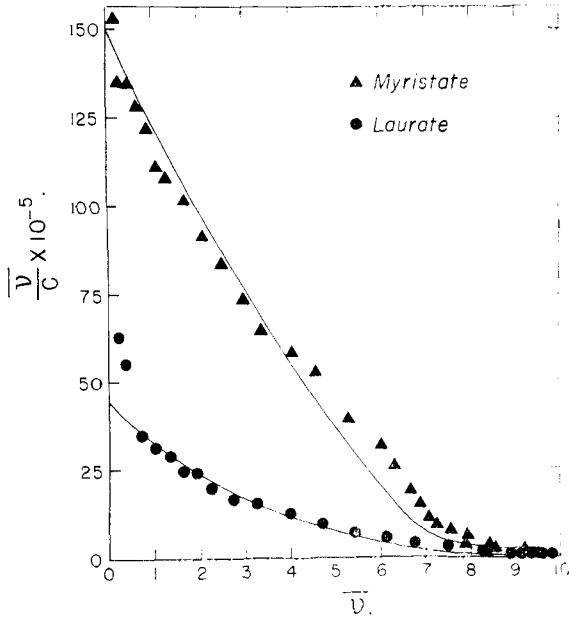

Fig. 4.

were turbid after equilibration with concentrations higher than those plotted in Figs. 2 and 5. Finally, the values of k_3' are highly inaccurate and should be considered merely as approximations.[35,36]

Relation of Apparent Constants to Intrinsic Constants.—The apparent association constants herein determined differ from the intrinsic association constants for the interaction of serum albumin with fatty acid anions in several ways.

In the first place, these experiments were conducted in phosphate buffer, and the possibility

(35) The only value of k_3' which has any accuracy is that for linoleate, since this was the only ion where values of $\bar{\nu}$ greater than 10 were observed (up to 13.5).

(36) In addition, the possibility that there is a fourth larger class of still weaker binding sites, consisting of the remaining cationic residues on the albumin molecule, should be mentioned. These sites, if present will never be detectable for the long-chain fatty acid anions because of the limited solubility of these materials.

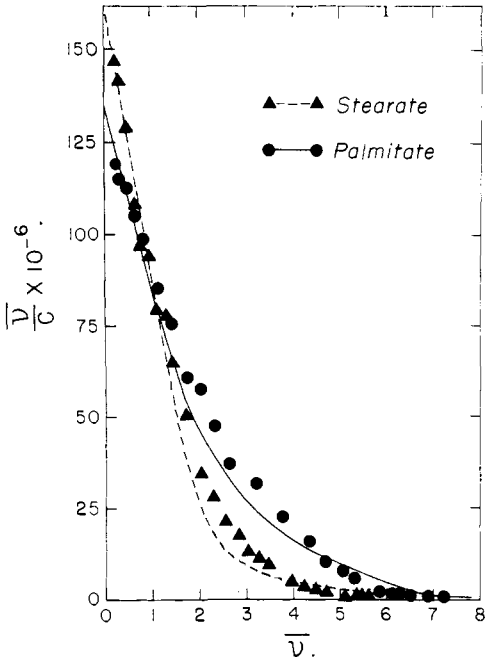

Fig. 5.

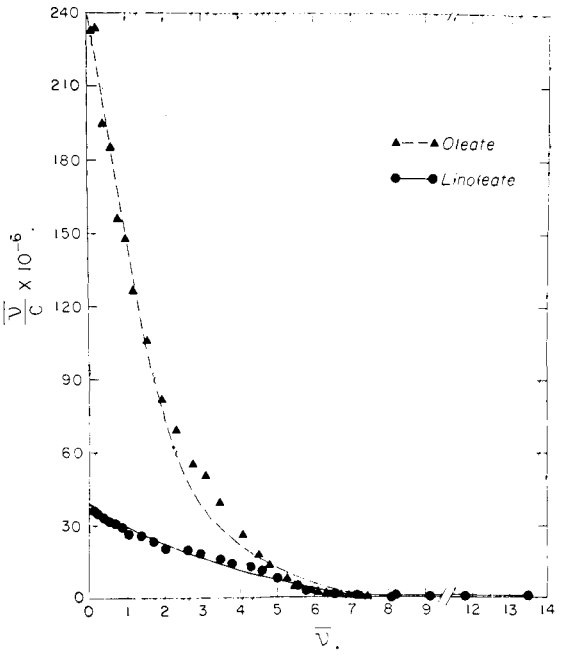

Fig. 6.

exists that phosphate ions can compete with fatty acid ions for the same binding sites. Scatchard has shown[31] that when several small ions (A, B, D, ... etc.) compete for binding sites on a protein, the

TABLE I

THE APPARENT ASSOCIATION CONSTANTS FOR THE INTERACTION OF HUMAN SERUM ALBUMIN WITH FATTY ACID ANIONS AT *p*H 7.45, IONIC STRENGTH 0.160, 23°, WITH THREE CLASSES OF BINDING SITES, $n_1 = 2$, $n_2 = 5$ AND $n_3 = 20$

Fatty acid anion	k_1'	k_2'	k_3'
Laurate	1.6×10^6	2.4×10^5	6×10^2
Myristate	4.0×10^6	1.4×10^6	2×10^2
Palmitate	6.0×10^7	3.0×10^6	1×10^3
Stearate	8.0×10^7	8.0×10^5	1×10^3
Oleate	1.1×10^8	4.0×10^6	1×10^3
Linoleate	1.3×10^7	2.5×10^6	2.5×10^3

apparent association constant k'_{Ai} is related to the association constant in the absence of such competition k_{Ai} by the equation

$$k'_{Ai} = \frac{k_{Ai}}{1 + k_{Bi}c_B + k_{Di}c_D + \ldots} \qquad (2)$$

The difference between k'_{Ai} and k_{Ai} might be quite large if competition actually occurred in the present studies. Thus if one assumed that phosphate ions compete with fatty acid ions and that phosphate binding is similar in magnitude to chloride binding the application of equation 2 would indicate that k_1 is roughly $100 \times k_1'$ and k_2 roughly $5 \times k_2'$.[37] Whether such competition occurs is, of course, not known, and no attempt has therefore been made to correct the apparent association constants (Table I) for competition with buffer ions. It should be pointed out parenthetically that the experimental technique employed in these studies requires the presence of a buffer, for the values of the partition ratio are highly sensitive to *p*H changes.

A second difference between the apparent and the intrinsic association constants derives from the fact that the fatty acid anions probably bind to cationic sites on the albumin molecule and that the availability of such cationic sites will depend on their equilibrium with hydrogen ions. Saroff[38] has shown that when this is the case the apparent association constant should be corrected by the expression

$$k'_{Ai} = \frac{k_{Ai}k_{Hi}c_H}{1 + k_{Hi}c_H} \qquad (3)$$

where k_{Ai} is the corrected apparent association constant, k_{Hi} is the association constant for H^+ binding to the group to which A also binds, and c_H is the concentration of hydrogen ions. Equation 3 is of such form that this correction factor is significant only when $k_{Hi}c_H$ is very small (*i.e.*, is one or less). Thus, for these studies at *p*H 7.45, if the cationic sites to which fatty acid ions bind are the ϵ-amino groups of lysine (pK^0_H 9.8)[39] this correction factor is not significant (less than 1%). If, however, the important binding sites are the ionized imidazolium groups of histidine (pK^0_H 6.9) the correction factor would be significant, for in this case k_{Ai} would be of the order of $4 \times k'_{Ai}$, and, in fact, the values of n might be sensitive to *p*H changes. Since the nature of the sites to which fatty acid ions bind is not known, whether or not this correction is applicable to the present studies cannot be decided at this time.

A final difference between the apparent and intrinsic association constants arises from electrostatic interactions between the fatty acid anions and

(37) This rough estimate neglects consideration of electrostatic effects, in using Scatchard's intrinsic constants for chloride binding,[32] and should be considered only as indicating the possible order of magnitude of the effects involved. If electrostatic effects are considered the correction factors will be smaller.

(38) H. A. Saroff, *J. Phys. Chem.*, **61**, 1364 (1957).

(39) C. Tanford, S. A. Swanson and W. S. Shore, THIS JOURNAL, **77**, 6414 (1955).

the albumin molecules. Using a simple spherical protein model with a uniform distribution of charge on its surface, Scatchard[30] has shown that

$$k_{Ai} = k^0_{Ai} e^{-2w z_p z_A} \quad (4)$$

where k_{Ai} is the association constant corrected for effects of competition, *et al.* (discussed above), k^0_{Ai} is the intrinsic association constant, z_p is the net charge of the protein, z_A is the charge of A and w is the usual Debye–Hückel parameter. This theory recently has been modified by Tanford and Kirkwood[40] using a model in which the charges on the protein are taken to be discrete unit charges located at fixed positions and by Saroff[38] using a model in which hydrogen bonding is assumed to occur between carboxylate and cationic nitrogen groups on the albumin molecule. Electrostatic corrections have not been applied to the results of the present studies for two reasons. First, in order to apply the proper correction one would have to know whether fatty acid ions compete with phosphate ions and, in fact, whether they compete with $HPO_4^=$ or $H_2PO_4^-$ ions. If no competition occurs, the binding of a fatty acid ion would change z_p by -1; if, however, a fatty acid ion displaces a $H_2PO_4^-$ ion, this would result in no net change of z_p, whereas if it displaces a $HPO_4^=$ ion z_p would be changed by $+1$. Second, in order to estimate z_p correctly the binding of phosphate ions under these conditions would have to be known. The proper application of electrostatic corrections must hence await further knowledge about phosphate binding and about its competition with fatty acid binding.

In conclusion it should be restated that the association constants listed in Table I are the apparent constants for the conditions employed in these studies and might be quite different from the intrinsic constants for fatty acid ion binding. It should also be noted that the correction factors are such that the intrinsic constants will certainly be larger than the apparent constants. Finally, it should be noted that the conditions of these studies (*p*H 7.45, ionic strength 0.16) are almost the same as those which obtain in blood plasma of mammalian organisms[41] and that the apparent association constants can therefore be applied to *in vivo* studies involving fatty acid–serum albumin interactions.

Relation of Fatty Acid Structure to Binding Constants.—The most striking result of these studies is the observation of the extremely high affinity of the serum albumin molecule for the binding of up to two long-chain fatty acid anions. The values of k'_1 listed in Table I are considerably larger than the values of the association constants previously reported for the interaction of serum albumin with any small organic or inorganic ions. Part of this difference lies in the fact that almost all previously reported binding studies were performed with albumin preparations which had not been specially treated to remove tightly bound fatty acid. Such albumin preparations undoubtedly contained some tightly bound fatty acid, and it might well be that the first two sites were in this way obscured. This is only part of the explanation, however, for Scatchard, *et al.*, using resin deionized bovine mercaptalbumin (which contained slightly more than one mole of long-chain fatty acid per mole of albumin)[19] found that a number of small anions (Cl^-, I^-, SCN and CCl_2COO^-) bound to a single site much more tightly than to the rest and have reported the intrinsic association constant for trichloroacetate to this first binding site to be 4.6×10^4.[32] The values of k'_1 observed in the present study are two or three orders of magnitude greater than this, and the values of the intrinsic association constants would be even greater (see above discussion). It is hence apparent that the non-polar side chains of the fatty acids herein studied participate significantly in the binding process. That the non-polar portions of an ion contribute to its interaction with serum albumin has, of course, been known for some time. Thus Teresi and Luck[9] found that binding increased with chain length for the fatty acid anions acetate through caprylate, and Karush and Sonenberg[42] reported that the binding of alkyl sulfates was increased by an increase in the size of the non-polar part of the molecule. This effect of the non-polar portion of an ion usually has been ascribed to a summation of a number of short range non-specific van der Waals interactions between the non-polar portions of the binding ion and the non-polar side chains of the albumin molecule. The fact that the values of k'_1, for the homologous saturated fatty acids laurate through stearate, increase with increasing chain length is qualitatively consistent with this explanation. Closer inspection of the actual values of k'_1 listed in Table I, however, suggests that a great deal of structural specificity is involved. This is, in the first place, suggested by the magnitude of the values of k'_1. More convincing, however, is the observation of the very different effect on k'_1 of increasing the length of each fatty acid by two methylene units. Thus k'_1 for the 14C acid (myristate) is $2.5 \times k'_1$ for the 12C acid (laurate), whereas k'_1 for the 16C acid (palmitate) is $15 \times k_1$ for the 14C acid, and k'_1 for the 18C acid (stearate) is only $1.3 \times k'_1$ for the 16C acid. It would hence appear that the two binding sites in the first class are rather specifically constructed so as to be able to bind a 16C or an 18C fatty acid much more tightly than one of chain length smaller than 16C. Furthermore, adding one double bond to the 18C acid (*i.e.*, oleate) results in an increase in k'_1 of almost 40%, whereas adding a second double bond (linoleate) decreases k'_1 by a factor of almost 10 (as *cf.* oleate). Whether the oleate ion contains the optimal structure for interacting with the first class of binding sites cannot be decided without studying other structures (particularly fatty acids longer than 18C), but with the information available one could reasonably speculate that this might indeed be the case.

In contrast to k'_1 the values of k'_2 listed in Table I show a much smaller variation for the six fatty acids studied. Thus the values of k'_2 for palmitate, oleate and linoleate are all very close to each other, and, in fact, the differences between these three

(40) C. Tanford and J. G. Kirkwood, THIS JOURNAL, **79**, 5333 (1957).

(41) With the exceptions that chloride and bicarbonate, not phosphate, are the major anions of plasma and that *in vivo* temperature is 37° not 23°.

(42) F. Karush and M. Sonenberg, THIS JOURNAL, **71**, 1369 (1949).

constants are within the estimated error in the determinations of k'_2. k'_2 for myristate is approximately half this value, and k'_2 for laurate is approximately one-tenth this value. Also striking is the observation that k'_2 for stearate is less than k'_2 for palmitate. The estimated error in k'_2 is greatest for stearate, but it would appear that this difference is well outside this error. These results suggest that the second class of binding sites contains less structural specificity than the first class and that the structural specificity of this second class is different from that of the first class.

No comments can be made about the possible structural specificity of the third, weakest class of binding sites because of the large inaccuracy in the estimations of k'_3.

Finally, brief mention should be made of the question of whether the same sites which bind fatty acid anions are those which participate in the binding of a great many other anions to serum albumin. Scatchard, *et al.*,[32] found that the binding of chloride, iodide, thiocyanate and trichloroacetate ions could be expressed in terms of three classes of binding sites, with $n_1 = 1$, $n_2 = 8$ and $n_3 = 20$; they also reported that their results could be fitted, though not quite so well, by $n_1 = 2$, $n_2 = 6$ and $n_3 = 18$. The similarity between these values of n_1 and n_2 and those observed in the present study is quite suggestive, although any comparison is limited by differences in the protein preparations employed (see above). In addition, the studies of Karush on the competitive binding of *p*-(2-hydroxy-5-methylphenylazo)-benzoate and dodecyl sulfate ions to crystallized bovine serum albumin (in phosphate buffer *p*H 7.0), showed that one site, or possibly two, bound the latter tightly but not the former and that there were two other groups, one with 4 or 5 sites and one with about 17 sites, which bound both ions.[32,43] Unpublished studies from this Laboratory[44] also have shown that methyl orange anions bind to two groups of sites on fatty-acid-free human serum albumin (in phosphate buffer *p*H 7.45) with $n_1 = 4$, $n_2 = 18$, $k'_1 = 1.3 \times 10^5$ and $k'_2 = 2.2 \times 10^4$. The binding of methyl orange anions was not altered by the addition of 1.8 moles of long-chain fatty acid anions to the albumin, indicating that the first two sites which bind fatty acid ions so tightly are not available for the binding of methyl orange anions. One may hence reasonably speculate that the second and third classes of sites which bind fatty acid ions are also those to which many other anions are bound but that because of the greater structural specificity of the first class of sites, it is unavailable for the binding of relatively bulky ions like the dye ions. The first class of sites, however, might well be available for the binding of small simple ions (*e.g.*, Cl^-, I^-, SCN^-) and ions of structure similar to that of long-chain fatty acids (*e.g.*, dodecyl sulfate ions).

Implications for Metabolic Studies.—Recent studies of the unesterified fatty acids present in human blood plasma have indicated that these fatty acids are of great metabolic significance, in representing a transport form of lipid readily available as substrate for energy production. This conclusion is derived from studies of the arteriovenous differences,[12] the rapid turnover rates[45] and the responsiveness of the unesterified fatty acid fraction to changes of dietary status and hormonal administration.[11-13] The normal level of these fatty acids in humans is of the order of 5×10^{-4} mole per liter of plasma.[11,13] This level represents a $\bar{\nu}$ of fatty acid to albumin of slightly less than 1. Preliminary studies of the composition of this fatty acid fraction indicate that it consists of approximately 80% of oleic, palmitic, stearic and linoleic acids (in decreasing amounts), 5–10% palmitoleic acid and an isomer of oleic acid, 5–10% polyethenoid 20C acids and 5% of 14C to 10C acids.[46] Eighty-five to ninety per cent. of these acids are hence represented by the acids whose binding to albumin has been herein studied. The values of k_1' are such that for $\bar{\nu}$ of 1 the concentration of free (unbound) fatty acid anion is approximately 10^{-8} mole/liter. In normal human plasma, therefore, less than 0.01% of the unesterified fatty acids present are free in solution.[47] It thus appears that albumin is particularly well constructed to serve as a transport vehicle for fatty acids and that the albumin–fatty acid complex must be thought of as an entity with considerable biological significance.

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(43) F. Karush, THIS JOURNAL, **72**, 2714 (1950).

(44) D. S. Goodman, unpublished observations. Also see ref. 13.

(45) D. Fredrickson and R. S. Gordon, Jr., in preparation.

(46) V. P. Dole and A. T. James, unpublished observations. I am indebted to Dr. Dole for furnishing me this information.

(47) Recent studies in this Laboratory (D. S. Goodman and E. Shafrir, to be published) indicate that, with normal protein and fatty acid concentrations, some 1–2% of the unesterified fatty acids in plasma are bound to low density lipoproteins and that 1–2% are bound to red cells. It also appears that a similar amount are bound to high density lipoproteins. Under normal conditions, therefore, the relations between bound and unbound fatty acids may be considered identical with those which hold for purified systems containing only albumin. When the mole ratio of fatty acid to albumin ($\bar{\nu}$) is increased above 2, however, relatively more and more fatty acid becomes bound to lipoproteins, and the free fatty acid concentration is then smaller than that calculated on the basis of albumin binding alone.