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# Analysis of Long-Chain Free Fatty Acid Binding to Bovine Serum Albumin by Determination of Stepwise Equilibrium Constants*

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ABSTRACT: The stepwise equilibrium method was employed to analyze the binding of long-chain free fatty acids to bovine albumin. Equilibrium partition incubations were done at 37° in a calcium-free Krebs–Ringer phosphate buffer (pH 7.4). Charcoal-extracted crystalline bovine serum albumin and $^{14}C$-labeled fatty acids were used. In general, the 16-carbon atom acids were bound more tightly than either the 14- or 18-carbon atom acids. With all of the acids, we noted the presence of one very strong albumin binding site having an equilibrium constant in the range of $10^7$ $M^{-1}$ and two sites with constants in the range of $10^6$ $M^{-1}$. Five additional sites having constants ranging from $10^5$ to $10^4$ $M^{-1}$ also were detected. In every case the equilibrium constants for binding of the first 4 moles of fatty acid occurred in descending order: $K_1 > K_2 > K_3 > K_4$. This suggests that appreciable cooperative binding effects were not evident over the range of fatty acid:albumin molar ratios that are usually employed in metabolic studies. From partition data with a protein-free aqueous phase, the extent of aqueous dimerization was estimated for palmitic, stearic, and oleic acids. Anion dimerization corrections then were calculated for the corresponding binding data, assuming that only the fatty acid anion monomer interacts with albumin. A reanalysis of these corrected data revealed that little or no change was produced in the magnitude of the first four equilibrium constants. This indicates that anion dimerization has little effect upon the binding parameters when the fatty acid:albumin molar ratio is within the usual physiological range.

Long-chain free fatty acids (FFA)[1] are very poorly soluble in the aqueous media that are employed for most biological reactions. Therefore, a carrier is used in order to introduce sufficient amounts of FFA into the incubation medium or to take up FFA that is released in the course of a metabolic reaction. In the majority of cases, BSA is used experimentally as the FFA carrier or acceptor. Hence, it is important to have a thorough understanding of the interaction of FFA with BSA in order to interpret accurately many of these metabolic studies.

The binding of FFA to BSA has been investigated previously in some detail (Teresi and Luck, 1952; Boyer *et al.*, 1946a; Reynolds *et al.*, 1968; Spector *et al.*, 1969). These data were analyzed by a method that was based on the Scatchard binding model (Scatchard, 1949; Fletcher and Spector, 1968). Recently, it has been shown that the "apparent association constants" of the Scatchard model need not be meaningful biological parameters (Fletcher *et al.*, 1970). In fact the correction of the Scatchard parameters by means of the so-called "statistical factors" (Edsall and Wyman, 1958) gives true equilibrium constants only under the assumptions that each site is uninfluenced by its neighbor and each has the same intrinsic affinity for the ligand (Klotz, 1953). On the other hand, analysis of macromolecule–ligand interactions by a series of stepwise equilibria (Klotz, 1946; Klotz *et al.*, 1946) is sufficiently general to account for electrostatic interaction and other phenomenon that may be associated with FFA–

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† Recipient of a Research Career Development award from the National Heart and Lung Institute (K4-HE-20,338).

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[1] Abbreviations used are: FFA, free fatty acid; BSA, bovine serum albumin.

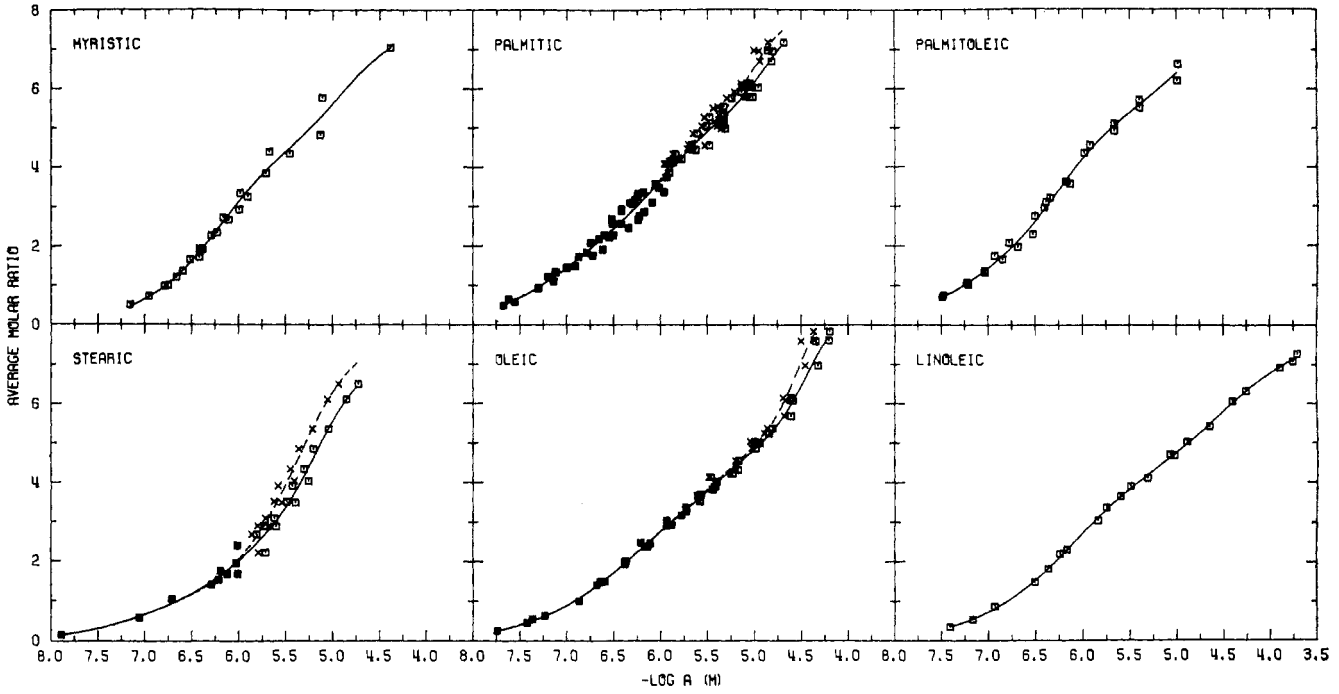


FIGURE 1: Graphs for fatty acid binding to bovine serum albumin. The squares represent data points in which the *total* unbound FFA concentration was used for the calculations, and the crosses, those in which the unbound FFA anion monomer concentration was used. The solid curve represents the calculated binding isotherm for the uncorrected data; the dashed curve for the unbound anion monomer data.

protein binding. Moreover, the actual equilibrium constants for the binding of each mole of ligand can be estimated directly by this procedure, whereas the Scatchard analysis, at best, provides only an average approximation of the association constants for a group of binding sites. The Scatchard model, however, is mathematically consistent with the stepwise equilibrium model and it can be expected to faithfully reproduce a binding isotherm. Thus, if one seeks detailed information concerning the nature of the respective association constants, the stepwise model rather than the Scatchard model should be used. Therefore, we have employed a method for fitting the binding data directly to a series of stepwise equilibrium reactions (Fletcher *et al.*, 1970).

This communication describes an analysis of long-chain FFA binding to BSA in terms of the stepwise equilibrium model. In addition, the effect of FFA dimerization in aqueous solutions (Mukerjee, 1965) on the binding of palmitic, stearic, and oleic acids has been examined.

## Methods

All of the materials and procedures employed in this study have been described in complete detail (Spector *et al.*, 1969). In summary, crystalline BSA purchased from Pentex, Inc. (Kankakee, Ill.), was incubated with charcoal in order to remove inherent FFA (Chen, 1967), dialyzed, and then dissolved in a medium containing 132 mM NaCl, 4.9 mM KCl, 1.2 mM $MgSO_4$, and 16 mM $Na_2HPO_4$. The solutions were adjusted to pH 7.4 with 0.1 N HCl. Purified fatty acids labeled with $^{14}C$ were mixed with carrier fatty acids obtained from the Hormel Institute (Austin, Minn.) and dissolved in *n*-heptane. Incubation was performed in specially constructed semimicro flasks containing Teflon-lined caps and a hollow sampling tube (Spector *et al.*, 1969). In one series of flasks 1 ml of heptane containing varying amounts of FFA was incubated with 1 ml of 0.2 mM BSA dissolved in the buffered salt solution. In a second series, 1 ml of FFA–heptane was incubated with 1 ml of the buffered salt solution containing no protein in order to measure the distribution (partition ratio) of FFA between the two phases (Goodman, 1958a). Incubation took place for 16 hr in a 37° water bath with shaking at 40 oscillations/min. The radioactivity contained in aliquots of the heptane and aqueous phases was measured in a liquid scintillation spectrometer using a toluene–methanol scintillator solution (Spector *et al.*, 1969). Quenching was monitored with the external standard. The experimental data were obtained by procedures previously reported (Goodman, 1958b; Spector *et al.*, 1969) and consisted of the total unbound FFA concentration in the molarity, [A], as a function of the molar ratio of bound FFA to albumin ($\bar{\nu}$). Corrections for anion dimerization in the aqueous phase were calculated from our partition ratio data for palmitic, stearic, and oleic acids (Mukerjee, 1965). In those instances where the "corrected" partition ratio data are used, the unbound anion monomer concentration in the aqueous phase [A′] is substituted for the total unbound FFA concentration.

The data consisting of experimental values of $\bar{\nu}$ for each value of A or A′ was fitted to the stepwise equilibrium model, eq 1, where the unknown parameters $K_1$, $K_2$, $\cdots$, $K_n$ represent

$$\bar{\nu} = \frac{K_1[A] + 2K_1K_2[A]^2 + \cdots + nK_1K_2\cdots K_n[A]^n}{1 + K_1[A] + K_1K_2[A]^2 + \cdots + K_1K_2\cdots K_n[A]^n} \quad (1)$$

the equilibrium constants for the binding of the ligand to the macromolecule. Starting estimates for the $K_i$'s are necessary in order to fit eq 1 directly to binding data by means of a data-fitting procedure. Preliminary estimates for the first fifteen stepwise equilibrium constants were calculated employing eq 22 and 23 of Fletcher *et al.* (1970) together with the parameters reported previously for FFA binding to BSA (Spector *et al.*,

TABLE I: Association Constants for FFA Binding to BSA.[a]

| Equilibrium Constants ($M^{-1}$) | Myristic | Palmitic | Palmitic[b] | Palmitoleic | Stearic | Stearic[b] | Oleic | Oleic[b] | Linoleic |
|---|---|---|---|---|---|---|---|---|---|
| $K_1 \times 10^{-7}$ | 0.76 | 3.20 | 3.28 | 3.13 | 1.30 | 1.30 | 1.65 | 1.65 | 1.10 |
| $K_2 \times 10^{-6}$ | 2.88 | 7.79 | 7.53 | 8.00 | 1.19 | 1.19 | 2.96 | 2.96 | 2.52 |
| $K_3 \times 10^{-6}$ | 1.50 | 3.48 | 3.67 | 2.91 | 0.67 | 0.48 | 1.77 | 1.77 | 1.28 |
| $K_4 \times 10^{-5}$ | 9.77 | 6.78 | 5.54 | 15.49 | 1.97 | 4.57 | 3.45 | 3.45 | 5.67 |
| $K_5 \times 10^{-5}$ | 2.85 | 8.78 | 12.27 | 12.14 | 1.46 | 2.07 | 1.94 | 2.11 | 1.35 |
| $K_6 \times 10^{-4}$ | 6.68 | 13.14 | 11.57 | 27.14 | 14.87 | 14.98 | 3.03 | 3.27 | 3.36 |
| $K_7 \times 10^{-4}$ | 8.23 | 2.01 | 2.47 | 6.96 | 6.74 | 13.46 | 2.25 | 2.32 | 2.18 |
| $K_8 \times 10^{-4}$ | 1.48 | 28.42 | 53.01 | 4.67 | 2.21 | 4.99 | 3.10 | 4.26 | 0.35 |
| $K_9 \times 10^{-4}$ | | | | | | | 1.66 | 4.34 | |
| Number of data points | 24 | 73 | 73 | 25 | 24 | 24 | 44 | 44 | 21 |
| Root-mean-square error | 0.176 | 0.204 | 0.219 | 0.131 | 0.212 | 0.216 | 0.124 | 0.142 | 0.049 |

[a] Incubation at 37° in media containing 0.2 mM BSA, 116 mM NaCl, 4.9 mM KCl, 1.2 mM $MgSO_4$, and 16 mM sodium phosphate (pH 7.4). [b] Binding data corrected for anion dimerization using the following constants: palmitate, $K_D = 2 \times 10^4$; stearate, $K_D = 7 \times 10^4$; and oleate, $K_D = 7 \times 10^3$.

1969). A computer program written in PL/1 performed these computations on an IBM System/360 computer. PL/1 was used because the compile-time preprocessor facilities of the language allow the program to be completely general so that any number of terms may be used in the computation of the preliminary estimates. Using these calculated estimates as starting values, the stepwise equilibrium model was refitted to the binding data for each fatty acid. The fitting was done using a least-squares model-fitting procedure in conjunction with an IBM 2250 graphic display unit and an interactive program called MODELAIDE (Shrager, 1970). The use of this procedure and equipment, including the graphical display, proved to be invaluable for the analyses of these data. This system enabled us to convert the rapid computer response directly into its most easily understood form, a graphical plot. The graphical effects of altering a parameter could be seen immediately at the display console where the user may control the program by a series of direct commands.

## Results

*Stepwise Equilibrium Analysis.* Figure 1 illustrates the experimental data points and the best-fitting binding isotherm calculated by the stepwise equilibrium method for the interaction of BSA with myristic, palmitic, palmitoleic, stearic, oleic, and linoleic acids. The equilibrium constants, $K_i$, for each of these binding isotherms are listed in Table I. A single very strong FFA binding site having a $K_1$ in the range of $10^7$ $M^{-1}$ was noted with each of the acids. The equilibrium constants $K_2$ and $K_3$ were in general of the same order of magnitude, $10^6$ $M^{-1}$.

Five or six additional sites were observed with equilibrium constants in the range of $10^5$–$10^4$ $M^{-1}$. The equilibrium constants for these sites could not be grouped in a manner that was consistent for all of the acids. The affinity of the first site for the FFA tested was palmitic > palmitoleic > oleic > stearic > linoleic > myristic. This differed somewhat from the affinity of the second site, and no consistent pattern of affinities was evident for the other binding sites except for the fact that either palmitic or palmitoleic acids were bound most firmly at sites 1–6.

In four of the cases the magnitude of the equilibrium constant for a given site was smaller than that for the next higher site: myristate, $K_6 < K_7$; palmitate, $K_4 < K_5$ and $K_7 < K_8$; oleic, $K_7 < K_8$. This reversal in the downward progression of $K_i$ values occurred only at relatively high $\bar{v}$ values, *i.e.*, where the data and hence necessarily the parameters are less accurately determined. Except in the case of palmitate, the magnitude of this reversal was quite small.

*Dimerization Effects.* The experimental data that have been presented so far are based upon the assumption that the total unbound FFA concentration in the aqueous medium is a reasonable approximation of the unbound FFA anion activity (Goodman, 1958b), the species that interacts with albumin (Boyer *et al.*, 1946a,b; Klotz and Walker, 1947; Teresi, 1950). This was assumed to be valid for incubations at pH 7.4 because the $pK_a$ of fatty acids is between 4.7 and 5.0 (Goodman, 1958a). However, a recent analysis of FFA distribution between *n*-heptane and phosphate buffer at 23° suggested the presence of aqueous dimerization of fatty acid anions (Mukerjee, 1965). The extent of anion dimerization was largest with palmitic, stearic, and oleic acids. In order to examine the effect of dimerization on FFA binding to BSA, we reanalyzed our partition results for these three acids by the method of Mukerjee.[2] The aqueous dimerization constants that we derived were: palmitate, $2 \times 10^4$; stearate, $7 \times 10^4$; and oleate, $7 \times 10^3$. Using these constants, the binding data with palmitate, stearate, and oleate were "corrected" by using the FFA anion monomer concentration instead of total FFA concentration. These corrected data were then refitted to eq 1 using the parameters corresponding to the uncorrected data as starting values. The data and fitted binding isotherms are shown in Figure 1. Data points representing the total unbound FFA concentration are shown as squares, while data points representing the unbound anion monomer concentration are shown as crosses. Likewise, the binding isotherm fitted to the uncorrected data (total unbound FFA concentration) is

[2] A complete analysis of our partition ratio results for each of these FFA will be presented in a separate communication. Some of the partition data have been illustrated graphically in an earlier report (Spector *et al.*, 1969).

illustrated by the solid curve; that fitted to the corrected data (unbound anion monomer concentration) is illustrated by the dashed curve. With each of the three acids, the difference between the uncorrected and corrected curves is quite small. Both the curves and data points are almost superimposed at the lower values of $\bar{\nu}$, and appreciable differences occur only at $\bar{\nu}$ values greater than 3–4. Equilibrium constants for the corrected data are listed in Table I. Comparison of these to the corresponding constants for the original data demonstrates further that the alterations resulting from these corrections are quite small, particularly for $K_1$–$K_4$.

## Discussion

Our results demonstrate that the binding of long-chain FFA to BSA can be analyzed in terms of multiple stepwise equilibria. The first binding constant, $K_1$, was considerably larger than the other constants for each of the acids considered. The second and third constants were of the same order of magnitude, but their ratios ranged from 1.7 to 2.7. The binding constants for the remaining sites did not exhibit behavior that was consistent for all the acids. Therefore, it appears that any grouping of the higher energy albumin binding sites into well-defined subclasses is somewhat arbitrary, particularly if one is attempting to define a model that is consistent for all of the physiologically important FFA. Previous analyses of long-chain FFA binding to serum albumin in terms of a Scatchard model suggested that three classes of binding sites were present (Goodman, 1958b; Fletcher and Spector, 1968). The models that have been proposed contain 2, 5, and 20 sites or 3, 3, and 63 sites. However, the present stepwise equilibrium analysis strongly suggests, at least for BSA, that it is more correct to either not subclassify the higher energy binding sites or to subdivide them in terms of a 1, 2, $m$ model. The latter interpretation is more consistent with the models proposed for thyroxine (Sterling, 1964) and coumarin (O'Reilly, 1967) binding to albumin.

Previous work with short- and medium-chain FFA (Teresi and Luck, 1952), anionic dyes (Klotz *et al.*, 1946), and detergents (Markus and Karush, 1957) indicated that BSA possessed a large capacity to bind organic ligands. Therefore, we believe that the relatively low binding capacity noted in the present experiments was not due to saturation of available BSA binding sites. On the other hand, it is likely that the maximum $\bar{\nu}$ values that could be reached experimentally were limited by the solubilities of the fatty acids in the aqueous incubation medium (Spector *et al.*, 1969).

In man, the FFA:albumin molar ratio rarely exceeds 4 and, for the most part, is between 0.5 and 2.0 (Fredrickson and Gordon, 1958; Havel *et al.*, 1967). Therefore, in metabolic experiments the FFA:albumin molar ratio is usually maintained within this range. Our results suggest that observable cooperative binding effects do not occur in this low $\bar{\nu}$ range. Cooperativity has been noted at higher $\bar{\nu}$ values, at least for binding of anionic detergents (Lovrien, 1963; Reynolds *et al.*, 1967, 1970). Some reversal in the downward progression of the FFA binding constants was found with some of the acids at values of $\bar{\nu}$ between 4.0 and 8.0. However, the low magnitudes of these reversals and the inherent uncertainty of the data at the higher $\bar{\nu}$ values makes the detection of true cooperative effects with FFA in this $\bar{\nu}$ range open to question.

Corrections of the experimental data for unbound anion dimerization (Mukerjee, 1965) produced only relatively minor changes in the binding results. Hence, dimerization appears not to be an important factor when the FFA:albumin molar ratio is within the usual physiological range. The dimerization constants calculated from our partition data are considerably smaller than those derived by Mukerjee (1965). This may reflect in part the fact that our conditions of incubation differed from those of Mukerjee. If Mukerjee's constants were applied to our binding data, the aqueous dimerization corrections would have been considerably larger. However, we feel that such an analysis is not justified because of the difference in experimental conditions.

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