Interactions of Long-Chain Fatty Acids and Albumin: Determination of Free Fatty Acid Levels Using the Fluorescent Probe ADIFAB†

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ABSTRACT: Equilibrium binding of long-chain fatty acids (FA) with albumin from human serum (HSA), bovine serum (BSA), and murine serum (MSA) has been studied by measuring the equilibrium levels of free fatty acids (FFA). FFA levels were measured directly, using a new fluorescent probe composed of acrylodan-derivatized intestinal fatty acid binding protein (ADIFAB). Measurements of [FFA] were done as a function of the ratio of total FA to total albumin (ν) for ν values between 0 and 6, at pH 7.4 and 37 °C. Under conditions observed in normal human physiology ($\nu \leq 2$), [FFA] values of the most abundant serum FA (palmitate, stearate, oleate) in equilibrium with human or bovine albumin are less than 15 nM. These values are considerably smaller than the generally quoted values of [FFA] in equilibrium with albumin: more than 20-fold for palmitate and more than 50-fold for oleate. FFA levels were found to increase monotonically with for all three albumins and all FA. In most cases [FFA] increased, for the same chain length, with increasing degree of acyl chain unsaturation, suggesting that FA aqueous solubility may play a significant role in the equilibrium between FA association with albumin and the aqueous phase. [The highest FFA levels (~3000 nM), for example, were observed for linolenate (18:3) at the maximum ν value (6).] Although aqueous-phase solubility of the FA may be important in understanding the interaction between FA and albumin, protein structure, as reflected in differences among the three albumins, also significantly affects the equilibrium. For example, overall, affinities for murine albumin were lower (higher [FFA] values) than for human and bovine albumins. Specific differences were also observed in the relative affinities of the three albumins for different FA. Oleate binds more tightly than palmitate to human and murine albumins, while the reverse is true for bovine. The measured binding isotherms are in all cases well described by a multiple stepwise equilibrium model with six association constants. In most cases, the association constant for the first site is greater than or equal to that of succeeding sites. In a few instances, however, in particular for arachidonate binding to human albumin, the results (a smaller constant for the first than the second site) suggest that binding may involve positive cooperativity. It is also shown that the binding isotherms generated by the multiple stepwise model, are themselves well described by a linear plus exponential function, thereby allowing FFA levels to be estimated by simple calculation.

Fatty acids (FA)[1] are the major source of physiologic energy. To provide this energy, FA are released from triacylglycerol stored in adipocytes or lipoproteins and are then transported through the blood to appropriate tissues. Most FA within the blood are associated with serum albumin, thereby allowing transport of large quantities of these relatively insoluble molecules (Spector, 1975; Spector & Fletcher, 1978; Kragh-Hansen, 1981). Although most FA are bound to albumin, a small fraction dissociates from the protein and exists in monomeric form within the aqueous phase. This quantity is the free fatty acid (FFA).[2] The interaction of FA and albumin serves to buffer the level of FFA and therefore regulates the rate at which FFA is transported to appropriate target cells. Although FA also bind to other blood components, serum levels of FFA are determined principally by FA binding to albumin because the buffering capacity of albumin greatly exceeds that of any other blood components (Spector & Fletcher, 1978). To gauge serum FFA levels, therefore, it suffices to consider the binding equilibrium between FA and albumin.

In addition to its FA transport role, albumin's capacity to buffer FFA levels is important because FFA are potent modifiers of cellular behavior. *In vitro* studies have shown that elevation of FFA levels can affect a wide variety of functions ranging from ion transport to cell adhesion (Spector & Fletcher, 1978; Karnovsky et al., 1982; Anderson & Welsh, 1990; Huang et al., 1992). Moreover, *in vivo* studies suggest that elevation of serum FFA is associated with diseases such as cancer (Brown et al., 1983; Legaspi et al., 1987), diabetes (Reaven et al., 1988), and ischemic injury (Burton et al., 1986; Jones et al., 1989; Ford et al., 1991), and elevated FFA levels may modulate the immune response in cancer patients (Brown et al., 1983). The *in vivo* studies suggesting a role for elevated FFA levels in immune suppression are complemented by *in vitro* studies by ourselves and others demonstrating that acute exposure to elevated levels of cis unsaturated FFA specifically inhibits cytotoxic T-lymphocyte activity (Samlaska, 1979; Taylor et al., 1985; Richieri & Kleinfeld, 1990). Whether such effects are physiologically significant *in vivo* will depend upon the serum FFA levels in disease, and a correlation of physiologic effects with *in vitro* results would

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[1] Abbreviations: AA, arachidonate; ADIFAB, acrylodated I-FABP; BSA, bovine serum albumin; cmc, critical micelle concentration; FA, fatty acid; FFA, free fatty acid; HSA, human serum albumin; I-FABP, intestinal fatty acid binding protein; LA, linoleate; LNA, linolenate; MSA, murine serum albumin; OA, oleate; PA, palmitate; SA, stearate.

[2] The term free fatty acid (FFA) is used to designate the aqueous-phase monomer of either the acid, the sodium salt, or the ionized molecule. Since the pK_a of the aqueous-phase monomer is 4.8 (Small, 1986), most of the molecules are ionized at the pH (7.4) used in this study. The term fatty acid (FA) is used to refer to the protein bound (nonfree) state of the molecule. Total FA (FA_t) equals FFA + FA.

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require that FFA levels found to modulate cellular responses *in vitro* are similar to those occurring *in vivo*.

Previously, FFA levels of long-chain FA have not been measured directly in serum, in equilibrium with albumin, or in cellular studies. Values have, however, been estimated using FA–albumin association constants determined in a series of careful measurements of the distribution of FA between a water–albumin and a heptane phase (Spector et al., 1971; Ashbrook et al., 1975). In these studies the distribution of radiolabeled FA was determined in the water–albumin and heptane phases as a function of the FA concentration, and the results were analyzed in terms of multiple stepwise equilibrium binding with up to 12 binding sites per albumin. Using the measured equilibrium constants and the equations for multiple stepwise equilibrium, [FFA] values have been calculated as a function of the ratio of total FA to albumin (Abumrad et al., 1981; Potter et al., 1989).

FFA levels estimated in this manner have been used extensively for interpreting *in vivo* and *in vitro* results (Spector & Fletcher, 1978; Abumrad et al., 1981; Potter et al., 1989). In particular, FA/albumin complexes using bovine serum albumin (BSA) have been used in *in vitro* studies to provide defined concentrations of FFA (Abumrad et al., 1981, 1991; Potter et al., 1989; Anderson & Welsh, 1990). Two potentially important problems may arise in this procedure. First, FFA levels predicted by these methods for long-chain FA have not been verified by direct measurement of [FFA] in equilibrium with the albumin–cell systems used in these studies. Second, [FFA] is an exponential function of the ratio of total FA to albumin (ν) for values of ν that exceed $\sim$3 (Spector et al., 1971; Ashbrook et al., 1975; and below). For these large ratios, small errors in ν result in large errors in the estimated [FFA] values, making extremely difficult the determination of the critical FFA levels. For example, cis unsaturated FFA inhibit the ability of cytotoxic T-lymphocytes (CTL) to kill target cells (Richieri & Kleinfeld, 1990). While complete inhibition of CTL-mediated killing of target cells occurs at oleate/albumin ν values $\sim$5.5, lysis of either CTL or target cell occurs at 6.0, making it difficult to differentiate between the fairly subtle inhibitory effects of CTL signaling caused by cis FA from the direct lytic effects of high concentrations of FFA.[3] In addition to illustrating this technical difficulty, these CTL studies also suggested that FFA levels predicted by the BSA association constants (Spector et al., 1971) were too high. Inhibition of CTL function achieved by direct addition of the acid or sodium salt of the FA in the absence of albumin was found to occur at free oleate <0.3 μM (Anel et al., 1993), whereas with oleate/BSA complexes, CTL inhibition requires ratios of >5:1, where the predicted values exceed 10 μM.

To resolve these issues, we have recently developed a method to measure directly [FFA] for the physiologically relevant long-chain FA. This has been done using a fluorescent probe composed of fatty acid binding protein from rat intestine (I-FABP) derivatized with the fluorescent molecule acrylodan and is referred to as ADIFAB (Richieri et al., 1992). The fluorescence emission of ADIFAB undergoes a shift from 432 to 505 nm upon binding a single FA, and therefore the ratio of emission intensities can be used to provide accurate values for [FFA] for a wide range of concentrations and for physiologic molecular species of FA. Under the conditions of these measurements neither ADIFAB fluorescence nor its equilibrium binding constants for FA are affected by the presence of other macromolecules, and therefore it is well suited to monitoring FFA concentrations in equilibrium with albumin, serum, or cells.

In the present study ADIFAB has been used to determine FFA levels for several of the physiologically important long-chain FA, buffered by human (HSA), bovine (BSA), and murine (MSA) albumins. The present results, which are the first direct measurements of [FFA] in equilibrium with albumin, yield values more than 20-fold lower than those predicted by the previous study of BSA (Spector et al., 1971) but are in reasonable agreement with the predictions of the previous study of HSA (Ashbrook et al., 1975). In addition, the present results are the first to describe the binding of FA to MSA.

EXPERIMENTAL PROCEDURES

Materials. Radiolabeled FA were from New England Nuclear, Boston, MA. Buffer in most studies was 140 mM NaCl, 5 mM KCl, 20 mM Hepes, and 1 mM NaH_2PO_4 at pH 7.4. Fatty acids and their sodium salts were purchased from Nucheck Prep (Illysian, MN). Measurements were done using several different lots of essentially fatty acid free albumin purchased from Sigma, and all lots gave virtually the same results. Thin-layer and gas chromatography as described previously (Richieri & Kleinfeld, 1991) was used to assess FA purity and the FA composition of the albumins. FA purity was consistent with that quoted by Nucheck Prep, and the FA content of the albumins was less than 1% per mole of protein. The FFA probe ADIFAB was prepared from acrylodan-derivatized recombinant rat intestinal fatty acid binding protein (I-FABP) as described (Richieri et al., 1992) and is available from Molecular Probes, Eugene, OR.

FA Handling and FFA Treatment. Sodium salts of the unsaturated, especially the polyunsaturated, FA were found to oxidize when maintained in the solid form, even at –20 °C and purged with argon. Therefore, upon arrival, the sodium salt of each FA was dissolved at a FA concentration of 20–50 mM in deionized water plus 4 mM NaOH and 25 μM butylated hydroxytoluene (BHT). Addition of sufficient NaOH (pH >9) at temperatures above the FA melting points (see below) produces an optically clear dispersion of the sodium salts of the FA, which we assumed to be a true solution. BHT was dissolved in EtOH at 50 mM, and a small volume (resulting in 0.05% ethanol) of this stock was added to the aqueous FA–sodium salt solution. Appropriate dilutions of these stock solutions were made in water plus 4 mM NaOH but no additional BHT (BHT was found to bind to ADIFAB with a K_d of about 10 mM). All stocks were maintained under argon at –20 °C for long-term storage but in no case for longer than 3 months.

Fluorescence Measurements. Measurements of the 505/432 intensity ratios (R values) were done with an SLM 8000C fluorometer using the photon counting mode with excitation (386 nm) slits set at 4 nm and emission slits at 8 nm. With these settings ADIFAB (0.5 μM) intensities were generally about 1×10^5/s at 432 nm and 2×10^4/s at 505 nm. Intensities ($< 2 \times 10^2$/s) of a blank sample (without ADIFAB) were integrated for 10 s. For measurements of the samples containing ADIFAB, 10 pairs of intensities were collected with 2-s integrations at 432 and 505 nm. After subtraction of the blank intensities, the average and standard deviation of the R value were determined. Typically, this standard deviation was <0.3% of the R value. Stopped-flow measurements were done using a HiTech SF11A device connected to the SLM8000C.

[FFA] Determination with ADIFAB. ADIFAB responds to FA binding by undergoing a shift in fluorescence from 432

[3] A. Anel and A. M. Kleinfeld, private communication.

nm in the apo to 505 nm in the holo form (Richieri et al., 1992). As a consequence, [FFA] can be determined from the ratio of 505- to 432-nm fluorescence by

$$[\mathrm{FFA}] = K_d Q(R - R_0)/(R_{max} - R) \quad (1)$$

where R is the measured ratio of 505- to 432-nm intensities (with blank intensities subtracted), R_0 is this ratio with no FFA present, R_{max} is the value when ADIFAB is saturated, $Q = I_F(432)/I_b(432)$, and $I_F(432)$ and $I_b(432)$ are the ADIFAB intensities with zero and saturating concentrations of FFA, respectively. Values of Q and R_{max} were found to be 19.5 and 11.5, respectively (Richieri et al., 1992), and equilibrium constants (K_d) were determined as described (Richieri et al., 1992; Anel et al., 1993). R values measured at pH 7.4 with the SLM 8000C range from approximately 0.26 with zero FA present (R_0) to about 6.0, where, for most FA, the [FFA] begins to exceed the critical micelle concentration (cmc).

[FFA] Determination in FA/Albumin Complexes. ADIFAB was used to determine FFA levels in the presence of FA/albumin complexes. For each complex, FFA levels were determined by measuring the fluorescence from three separate samples containing (1) albumin in buffer (blank), (2) albumin and ADIFAB in buffer (the R_0 value), and (3) FA, albumin, and ADIFAB in buffer. The same albumin (4–10 μM) and ADIFAB (0.5 μM) concentrations were used in each sample, and the total volume of the samples was 1.5 mL. Just prior to FA addition, the concentrated stocks of the sodium salts were warmed to temperatures above the FA melting point (72 °C for stearate, 62 °C for palmitate, and 37 °C for the others). FA were then added in small volumes to sample 3, which was maintained at 37 °C and immediately mixed by drawing the solution in and out of a pipet. Between each FA addition the cuvette was allowed to incubate for 10 min at 37 °C. After the 10-min incubation, the 432- and 505-nm intensities were determined from the three samples all measured at 37 °C. This procedure was repeated for ν values between 0 and 6.0, in steps of ~0.5. As an alternative, FA/albumin complexes were prepared using the acid form of FA adsorbed to diatomaceous earth (Celite) (Spector et al., 1969). FA with trace [^{3}H]-FA was added from ethanol to Celite (at 0.1 μmol of FA/mg of Celite), the ethanol was evaporated under argon, and a buffered solution of albumin was added to the dried FA/Celite mixture. After incubation for 1 h at 37 °C and removal of Celite by centrifugation, [FFA] was determined by adding ADIFAB to one sample of the FA/albumin complex and radioactivity of a duplicate sample was determined to establish the ν value of the complex. Both the direct FA titration of albumin with sodium salts of the FA (the method used for all of the data here) and the Celite procedure yielded virtually identical results.

ADIFAB itself has little effect on [FFA] in these measurements because of the FA binding capacity of albumin and because [albumin] and [FA_{total}] are much greater than [ADIFAB]. Relatively little of the total FA ([FA_t]) binds to ADIFAB. For example, with [HSA] = 4 μM, ν = 1.0, and [ADIFAB] = 0.5 μM, the ADIFAB measurement yields [FFA] ~5 nM for oleate (Figure 1). These conditions correspond to 7.5 nM FA bound to ADIFAB [eq 2 of Richieri et al. (1992)], and therefore less than 0.2% of [FA_t] (4 μM) is removed from the FA/albumin complex. Even if conditions were chosen so that ADIFAB binding was significant, [FFA] and the amount of FA bound to ADIFAB are correctly given by eqs 1 and 2 of Richieri et al., (1992), and therefore the effective ν value for albumin could be determined.

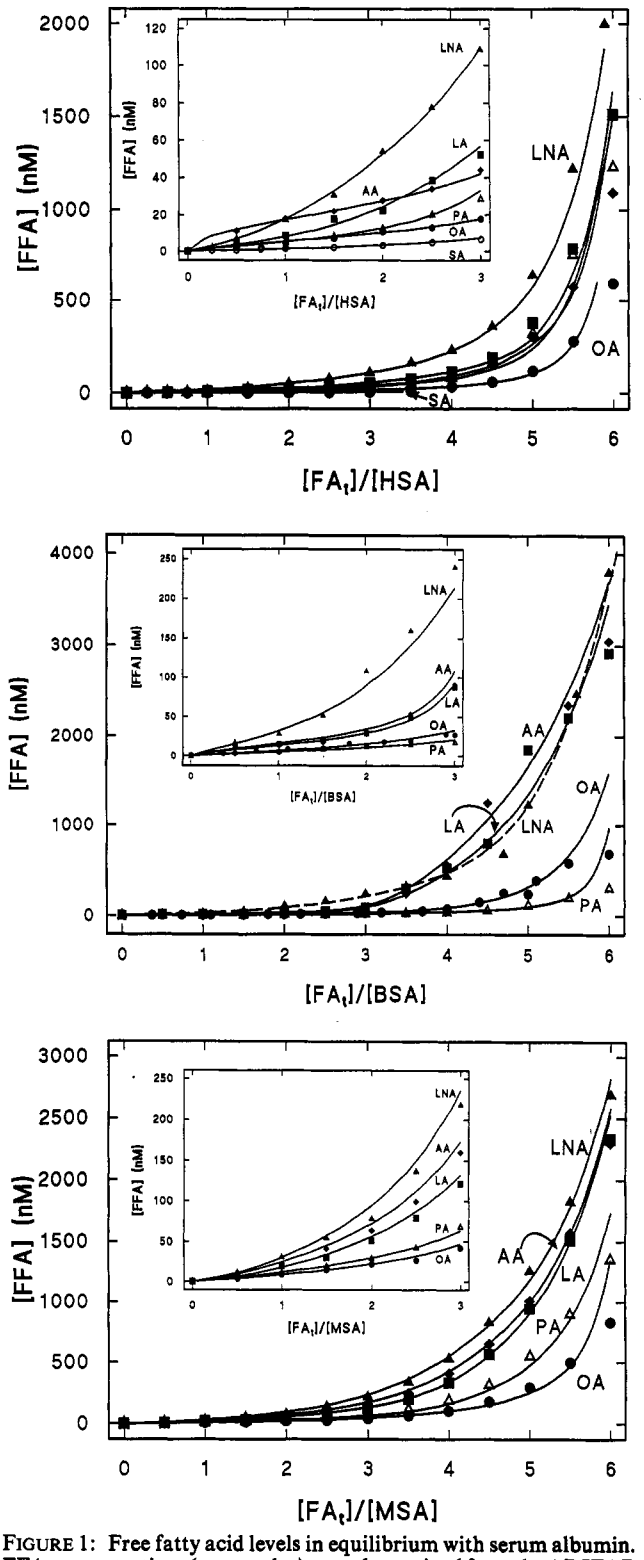

FIGURE 1: Free fatty acid levels in equilibrium with serum albumin. FFA concentrations (nanomolar) were determined from the ADIFAB fluorescence ratios measured in aqueous solutions (pH 7.4) of [ADIFAB] (0.5 μM), [albumin] (between 4 and 8 μM), and total FA (FA_t) titrated between 0 and 36 μM at 37 °C. Each measured [FFA] value, represented by the symbols, is an average of three or more separate determinations. The FA molecular species are represented by the following symbols: palmitate (△); stearate (○); oleate (●); linoleate (■); linolenate (▲); arachidonate (◆). Lines through the data points represent the multiple stepwise equilibrium analysis using equation 3 with the association constants of Tables I–III to calculate [FFA] values as described under Experimental Procedures. An expanded view of the results between ν = 0 and 3 is shown in the inset. (A) HSA; (B) BSA; (C) MSA.

R values measured with ADIFAB alone or in the presence of membranes, cells, or a variety of proteins is constant to

Table I: FA–HSA Association Constants[a]

fatty acid	K_a^1	K_a^2	K_a^3	K_a^4	K_a^5	K_a^6
palmitate	145	130	71	12	8	3
stearate	689	515	130	120	70	1
oleate	118	118	80	55	23	6
linoleate	135	82	18	11	10	1.5
linolenate	95	22	11	8	3	1
arachidonate	11	78	60	15	14	2

[a] Association constants ($M^{-1} \times 10^{-6}$) were determined using the multiple stepwise equilibrium analysis (eq 2) as described under Experimental Procedures.

Table II: FA–BSA Association Constants[a]

fatty acid	K_a^1	K_a^2	K_a^3	K_a^4	K_a^5	K_a^6
palmitate	122	153	75	30	20	10
oleate	127	100	40	30	5	1.8
linoleate	45	50	50	2.5	1	0.4
linolenate	27	26	4.7	3.4	1.7	0.25
arachidonate	34	45	44	2.2	0.5	0.5

[a] Association constants ($M^{-1} \times 10^{-6}$) were determined using the multiple stepwise equilibrium analysis (eq 2) as described under Experimental Procedures.

Table III: FA–MSA Association Constants[a]

fatty acid	K_a^1	K_a^2	K_a^3	K_a^4	K_a^5	K_a^6
palmitate	110	37	24	15	2.2	1.9
oleate	134	52	36	15	5	5
linoleate	99	22	11	7	1	1
linolenate	61	11	9	2	1	0.8
arachidonate	66	15	11	3.5	1	1

[a] Association constants ($M^{-1} \times 10^{-6}$) were determined using the multiple stepwise equilibrium analysis (eq 2) as described under Experimental Procedures.

within 0.5% over times longer than 5 h (Richieri et al., 1992; Anel et al., 1993; and data not shown). In the presence of albumin, however, a very slow increase in R value of about 0.01/h is observed and corresponds, at low [FFA], to an apparent increase of about 5 nM/h (for oleate). This effect is eliminated by the procedure described above where parallel R_0 measurements (sample 2) are made at the same time as those with FA (sample 3) since the same time-dependent increase in R occurs in the absence and presence of FA (data not shown). The reason for this time dependence is under study but does not appear to be due to unreacted free acrylodan, a collision-dependent reaction between ADIFAB and albumin, or simply the availability of free sulfydryl groups.

Multiple Stepwise Equilibrium Analysis. The experimental results, ν values as a function of measured [FFA], were analyzed by the multiple stepwise equilibrium model as described by Spector and his colleagues (Ashbrook et al., 1975). According to this model ν can be expressed as

$$\nu = \sum_{i=1}^{i=6} i[\mathrm{FFa}]^i \prod_{j=1}^{j=i} K_a^j \Big/ [1 + \sum_{i=1}^{i=6} [\mathrm{FFA}]^i \prod_{j=1}^{j=i} K_a^j] \quad (2)$$

where in the present study six FA binding sites per albumin, each having association constants K_a^i, have been used. A least-squares procedure based upon the Marquarat algorithm was used to fit eq 2 to the data, using standard deviations of 10% for each ν value. A six-parameter fit of this kind exhibits many local minima. An iterative procedure using visual inspection of the fit to alter initial fit parameters was adapted, thereby facilitating convergence, as measured by χ^2 values. Although we have no assurance that these values correspond to a global minimum, the values listed in Tables I–III correspond to χ^2 values <1.1 and by visual inspection (see Figure 4, for example) provide an excellent representation of the data.

Using the set of six K_a^i values determined by this procedure, values of [FFA] were calculated for arbitrary [FA_t] and [$albumin_t$] using the algorithm of Abumrad et al. (1981) in which

$$[\mathrm{FA_t}] = [\mathrm{FFA}] + [\mathrm{albumin_t}] \sum_{i=1}^{i=6} i[\mathrm{FFA}]^i \prod_{j=1}^{j=i} K_a^j \Big/ (1 + \sum_{i=1}^{i=6} [\mathrm{FFA}]^i \prod_{j=1}^{j=i} K_a^j) \quad (3)$$

is solved for [FFA] by iteration. The [FFA] vs ν values that are generated by eq 3 were also fit by least squares, with the following (arbitrary) function:

$$[\mathrm{FFA}](\nu) = m\nu + b + ce^{k\nu} \quad (4)$$

This was done to provide a more convenient method to estimate FFA levels than does the iterative procedure using eq 3. The parameters m, b, c, and k determined by this analysis are listed in Table IV. While eq 4 provides a direct estimate of FFA as a function of ν, its accuracy is limited to conditions where [FFA] is, to a good approximation, a function of ν only, $\nu < 5$, and [albumin] $\gtrsim 5$ [FFA] (see Figure 3, for example).

RESULTS

Binding of Fatty Acids to Human, Bovine, and Murine Albumins. Binding of long-chain FA to human, bovine, and murine albumins was measured at 37 °C using ADIFAB to determine FFA levels as a function of the ratio (ν) of total FA to albumin. The results of these measurements (Figure 1) show that FFA levels increase monotonically with increasing ν. For all FA, with the exception of arachidonate, and all albumins, this increase in [FFA] is approximately linear with ν for small values of ν and increases approximately exponentially for larger values of ν. Although qualitatively this behavior is similar for all FA and albumins, the value of ν where the departure from linearity occurs and the magnitude of [FFA] depend sensitively on the FA and albumin molecular species. In general, for a given albumin and FA chain length, [FFA] increases with increasing degree of unsaturation. For example, with HSA and ν equal to 1.0 (the average physiologic value) [FFA] values are approximately 1.6, 4, 9, and 17 nM for stearate (18:0), oleate (18:1), linoleate (18:2), and linolenate (18:3), respectively. Similar variations were found for BSA and MSA and, as discussed below, reflect in part the increasing aqueous solubilities of the FA with increasing double bond number (Richieri et al., 1992).

Although the overall trend of FA binding is similar for all three albumins, significant differences do occur. Some of these differences are illustrated in Figure 2, where the bindings of oleate and palmitate to HSA, BSA, and MSA are compared. Two important differences are apparent in this figure. First, binding of oleate to HSA is virtually identical to the binding of palmitate to BSA, and the reverse is true for palmitate binding to HSA and oleate to BSA. Second, binding of palmitate, oleate, and other FA (not shown) to MSA is significantly weaker than to BSA and HSA. For example, at ν equal to 1.0, the free concentrations of oleate and palmitate are approximately twice ($\sim$10 nM) for MSA as compared to BSA and HSA, for which free palmitate and oleate are $\sim$5 nM. In addition to these differences, arachidonate exhibits what may be cooperative binding behavior to HSA and BSA but not to MSA (Figure 1). Moreover, the binding of LNA relative to other FA appears to be considerably stronger for BSA than for HSA or MSA (Figure 1B,C).

Table IV: Linear plus Exponential Fit Parameters[a]

	HSA				BSA				MSA			
fatty acid	*m*	*b*	*c*	*k*	*m*	*b*	*c*	*k*	*m*	*b*	*c*	*k*
palmitate	4.1	−0.89	0.61	1.20	4.4	−0.03	0.23	1.16	9.3	−0.43	0.88	1.24
stearate	1.7	−0.25	0.02	1.56								
oleate	5.0	0.34	0.015	1.72	6.5	−0.19	0.13	1.54	9.4	−2.0	0.59	1.17
linoleate	13.0	−4.9	0.52	1.22	6.0	1.0	0.6	1.65	12.2	−5.1	4.1	1.07
linolenate	25.0	−7.6	0.98	1.23	39.0	−14.	3.4	1.15	1.0	−28.0	27.0	0.76
arachidonate	11.0	5.4	0.02	1.82	1.0	−0.5	2.0	1.4	2.9	−11.5	14.0	0.86

[a] Parameters of the least-squares fit of the equation $[FFA](\nu) = m\nu + b + ce^{k\nu}$ to the multiple stepwise equilibrium results for HSA, BSA, and MSA. Using the parameters *m*, *b*, and *c* which have units of nM (*k* has no units), [FFA] is given in nM. In most cases, as seen for example in Figure 4, using these parameters eq 4 reproduces the multiple stepwise equilibrium results to better than 20%.

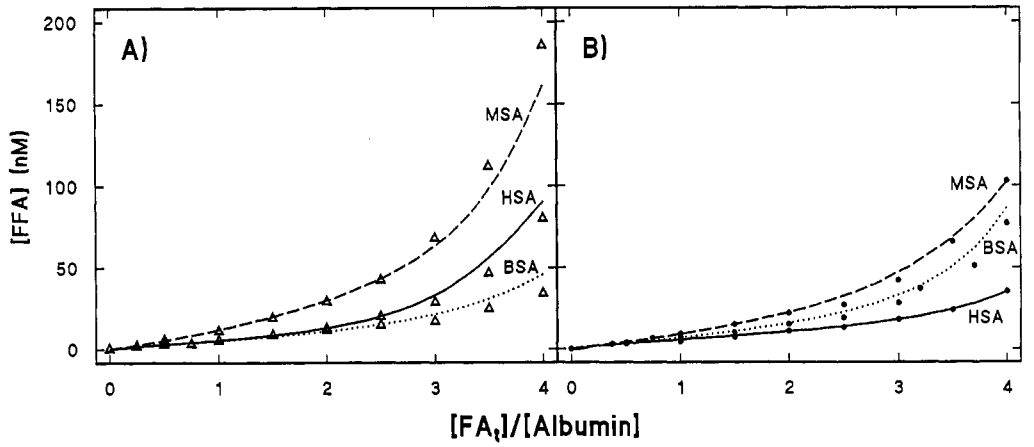

FIGURE 2: Comparison of FFA levels in equilibrium with HSA, BSA, and MSA. Data and multiple stepwise equilibrium results of Figure 1 for palmitate and oleate for all three albumins in the ν range between 0 and 4 are shown in (A) and (B), respectively. These plots illustrate the higher FFA levels of MSA compared to HSA and BSA and also illustrate "reversal" of palmitate and oleate values for HSA and BSA. Palmitate (△); oleate (●); HSA (—); BSA (···); MSA (- - -).

An important property of FA/albumin complexes and one that is predicted by multiple-site binding (see below) is that under most conditions [FFA] should be a function of ν only. Thus, although the [FA$_t$] may increase significantly, [FFA] will not change if ν is constant. This property of the complexes has been a source of some confusion. For example, as discussed by Potter et al. (1989), proper recognition of this property resolves a number of issues relating to cellular uptake of FA and in particular clarifies the role of FFA in FA transport. Using the ADIFAB method, this property was investigated directly by measuring [FFA] as a function of [albumin] for fixed values of ν. Results for linoleate/HSA complexes are shown in Figure 3. These results, which are similar to those for other FA/albumin complexes, show that for ν values ≤ 5, [FFA] is, to a good approximation, solely a function of ν for a wide range of [HSA]. At very low values of [HSA], [FFA] decreases to zero as [FA$_t$] decreases to zero. This relationship is not valid at higher ν values, reflecting the much weaker FA binding to higher order sites.

Fatty acids form relatively stable aggregates at concentrations exceeding their cmc values. The total FA concentrations ([FA$_t$]) used in the measurements illustrated in Figures 1–3 greatly exceed the cmc for these FA (Richieri et al., 1992). The free concentrations of FA in equilibrium with any of the albumins, however, are well below the cmc values and vary as predicted by the multiple equilibrium binding prescription (see below). Moreover, cmc values are reduced severalfold in the presence of calcium (Richieri et al., 1992), yet measurements of FA binding to BSA ($\nu \leq 6.0$) carried out in the presence of 1 mM $CaCl_2$ added to the standard buffer showed no difference from the zero calcium binding isotherms (data not shown). These results indicate that FA aggregates do not form in the presence of albumin. Therefore, association of FA with albumin greatly exceeds FA–FA association, even when binding principally involves the lower affinity binding sites, and calcium binding to albumin does not affect significantly the FA–albumin interactions.

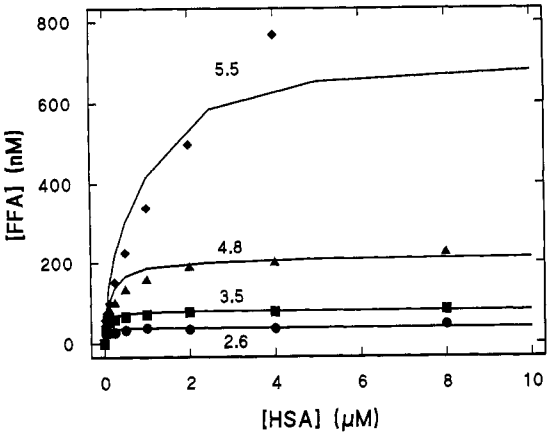

FIGURE 3: [FFA] values as a function of [HSA] for fixed ν values. Free linoleate values were measured using ADIFAB (0.5 M) at 37 °C in pH 7.4 buffer. In these measurements ν values ([LA$_t$]/[HSA]) were fixed at 2.6, 3.5, 4.8, and 5.6 and [HSA] was varied between 0 and 8 μM. The solid lines through the data are the predictions of the multiple stepwise equilibrium calculated using the association constants of Table I and eq 3.

Kinetics of FA Binding to Albumin. It is implicit that the binding isotherms shown in Figures 1–3 represent equilibrium values. The apparent affinities, however, are quite high, $K_a > 1 \times 10^8$ M^{-1}, raising the possibility that the off rate for FA dissociation from albumin might be slow on the time scale of these measurements (~10 min). To ensure that FA–albumin equilibrium was rapid, the quenching of the tryptophan fluorescence of albumin was measured, at 37 °C, upon FA binding (Spector & Fletcher, 1978). Using stopped-flow mixing, [FA$_t$] equal to 10 μM, and [BSA] equal to 5 μM, we

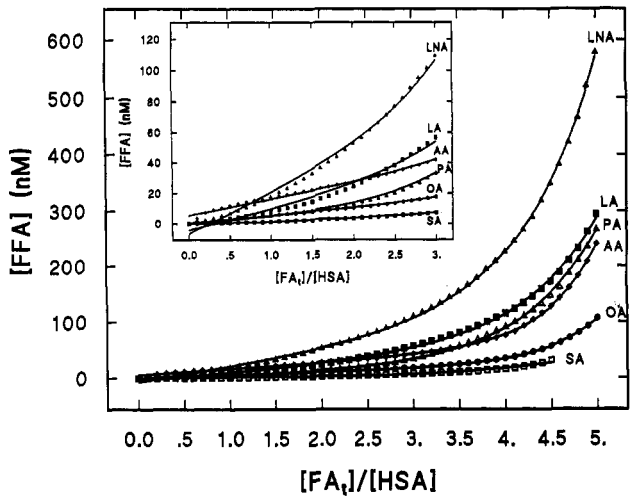

FIGURE 4: Linear plus exponential fit to the multiple stepwise equilibrium analysis for HSA. Symbols (with the same designation as in Figure 1) here represent multiple stepwise equilibrium values for the interaction of each FA with HSA. The lines through the data represent least-squares fits with a linear plus exponential function (eq 4) using the parameters shown in Table IV.

found that quenching (~20–30%) of tryptophan intensity was complete within the resolution (20 ms) of the instrument, indicating that equilibrium very likely occurred within this time (data not shown).

Multiple Stepwise Equilibrium Analysis. Previous FA binding and albumin structural studies have indicated that albumin contains a number of distinct FA binding sites (Spector et al., 1971; Ashbrook et al., 1975; Spector & Fletcher, 1978; Kragh-Hansen, 1981; Cistola et al., 1987a,b; Carter et al., 1989). Spector and his colleagues have demonstrated that FA/albumin binding isotherms can be well represented by a model in which binding of FA is represented by a multiple stepwise equilibrium mechanism with up to 12 association constants K_a^i per albumin (Spector et al., 1971; Ashbrook et al., 1975). A similar analysis was done in the present study, with the difference that the number of binding sites was limited to six. The results of these analyses are shown in Figures 1–3, and the complete set of affinity constants is listed in Tables I–III. These results show that the multiple stepwise equilibrium model (eq 2) using the six affinity constants determined from this analysis provides a good description of the binding isotherms. Excellent fits, as measured by χ^2, were obtained for the three albumins and all FA.

While it is unclear if the constants K_a^i in fact represent equilibrium constants for six discrete FA binding sites (Spector & Fletcher, 1978), these values do provide parameters that describe satisfactorily the multiple stepwise binding expression (eq 2). This in turn allows one to calculate FFA levels for arbitrary [FA] and [albumin] values using an appropriate iterative algorithm (Abumrad et al., 1981). As a means of simplifying further the procedure for calculating FFA levels as a function of ν, the binding isotherms predicted by stepwise equilibria were themselves parametrized, by least-squares analysis, with a function composed of the sum of linear and exponential functions of ν (eq 4). The parameters for each of these fits are also listed in Table IV. Using these functions, [FFA] may be calculated easily as a function of ν, and as seen in Figure 4 for HSA, these values provide good estimates of the values predicted by the multiple stepwise equilibrium analysis.

DISCUSSION

In this study, equilibrium binding of long-chain FA to human, bovine, and murine albumin was determined using the fluorescent probe ADIFAB to measure FFA levels. The results show that FFA levels increase monotonically with [FA$_t$]/[albumin] (ν) for the three albumins and all FA. The highest FFA levels (~3000 nM) occur at $\nu = 6$ for the polyunsaturated FA linolenate (18:3). Under conditions that more nearly represent those observed (Spector & Fletcher, 1978) in normal human physiology ($\nu \leq 2$), FFA levels are in the range 1–10 nM. Although overall the binding characteristics are similar for all three albumins, some qualitative and quantitative differences exist, including lower affinities for murine albumin than for human and bovine albumin and differences in the relative order of the HSA and BSA binding isotherms for oleate, palmitate, and linolenate. The binding isotherms measured are in all cases well described by a multiple stepwise equilibrium with six equilibrium constants. Moreover, these binding isotherms are themselves well described by a simple, albeit arbitrary, function that allows FFA levels to be estimated by a simple calculation.

Although FA binding to MSA has not been reported previously, the interactions of FA with BSA and HSA were studied previously by measuring the distribution of FA between heptane and the aqueous phase (Spector et al., 1971; Ashbrook et al., 1975). FA–albumin association constants were determined previously by a multiple stepwise equilibrium analysis (Spector et al., 1971; Ashbrook et al., 1975), and extensive use has been made of the K_a^i values for BSA to calculate FFA levels (Spector & Fletcher, 1978; Abumrad et al., 1981; Potter et al., 1989). However, [FFA] values for BSA obtained from the present study, using the K_a^i values of Table II, are more than 20-fold smaller, depending upon the molecular species and value of ν, than the results of the earlier study (Spector et al., 1971). It is likely that the values of K_a^i obtained from the multiple stepwise analysis for BSA in Spector et al. (1971) are at least 10-fold too small.[4] Indeed, an earlier Scatchard analysis of palmitate binding to albumin yielded association constants that were *greater* for BSA than for HSA (Spector et al., 1969). In contrast, the present results (Table I) for FA binding to HSA are in reasonable agreement with the previous study (Ashbrook et al., 1975).

Further support for the tighter FA binding to BSA (lower [FFA]) indicated by the present study is provided by the levels of cis FA required to inhibit the CTL response (Anel et al., 1993). In these studies, acute (seconds to minutes) exposure to elevated levels of cis unsaturated long-chain FA inhibits a variety of events in murine CTL. This inhibition was estimated to occur at free oleate concentrations <500 nM for CTL in the absence of albumin (Richieri & Kleinfeld, 1989, 1990; Richieri et al., 1990). Direct measurement of FFA levels with ADIFAB in CTL treated with inhibitory levels of FA has confirmed this estimate (Anel et al., 1993). When similar measurements are done using oleate/BSA complexes, a $\nu >$ 5:1 was required to achieve the same level of inhibition.[5] This is in good agreement with the predictions of the present study showing [free oleate] = 500 nM at $\nu = 5.5$ (Figure 1) but not with the earlier predictions of [free oleate] = 500 nM at $\nu \sim$ 2 (Spector et al., 1971).

The pattern of the molecular species dependence of FA binding to albumin shows, for fixed FA chain length, a sharp decrease in albumin association (increase in [FFA]) with the

[4] Dr. Arthur A. Spector, Department of Biochemistry, University of Iowa, Iowa City, IA, private communication.

[5] A. Anel and A. M. Kleinfeld, private communication.

number of double bonds. For example, for ν equal to 2.0 and n = 18, [FFA] increases by factors of about 14 for HSA (18:0–18:3), 4 for MSA (18:1–18:3), and 6 for BSA (18:1–18:3). Aqueous-phase solubility of FA increases with double bond number, and a similar pattern was observed for FA association with membranes and FABP (Richieri et al., 1992; Anel et al., 1993). This behavior suggests that, at least in part, FA aqueous solubility plays an important role in determining the relative equilibrium constants for FA association with albumin. While FA solubility likely plays an important role in determining the FA–albumin interaction, the overall pattern is modified by specific structural features of the proteins. For example, higher FFA levels for MSA indicate weaker overall FA–MSA interactions as compared to HSA and BSA, and the differences in oleate–palmitate interactions between HSA and BSA indicate different relative affinities for the two proteins. Specific FA–protein interactions are also suggested by the results of the multiple stepwise analyses. As discussed previously by Spector (1975), the decrease in K_a^i values with binding site order that is seen with most FA (Tables I–III) suggests that cooperativity, if it occurs, represents a relatively small contribution to binding of FA to albumin. In several cases, however, most especially for binding of AA to HSA and BSA, the K_a^i for binding to the first site is considerably smaller than the second (measurements of AA and LNA binding to albumin have not been reported previously). This raises the possibility, especially for AA, that there is significant positive cooperativity for subsequent binding for most of the other sites. These effects are unlikely to be due to unusual associations in the aqueous phase since AA binding to MSA does not exhibit this effect and binding of all FA to ADIFAB itself is well described by simple single site binding even at much higher [FFA] (Richieri et al., 1992; Anel et al., 1993). A general trend for most FA, suggesting more cooperative effects in HSA and BSA as compared to MSA, is also evident in the relative affinities for binding to the first two sites. Excluding AA, the average ratio of association constants of the first and second sites is about 1.5 for HSA and BSA and about 4 for MSA.

Although the multiple stepwise binding model provides an adequate representation of the FA–albumin interactions, the affinity constants determined from this analysis are unlikely to represent accurately actual equilibrium constants corresponding to unique FA binding sites. The minimization procedure using eq 2 is poorly behaved, and it is not known if the K_a^i obtained are those corresponding to a global minimum. No attempt was made in the present analysis to use more than six K_a^i values since adequate fits could be obtained with the limited range of data explored in this study. We did not, however, explore higher ν values where evidence for as many as 12 FA bound per HSA has been reported (Ashbrook et al., 1975) and therefore cannot exclude higher order binding sites. The structure of HSA at 6-Å resolution shows that HSA is composed of six different structural domains (Carter et al., 1989), thereby providing some justification for using six sites in the analysis. Moreover, the earlier Scatchard analysis of palmitate binding to BSA and HSA is consistent with six functional FA binding sites per albumin (Spector, 1975). In any event, either these K_a^i values together with the iterative method (eq 3) or the linear plus exponential function (eq 4) with the parameters of Tables I–IV provides accurate estimates of [FFA].

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