

Unbound free fatty acid levels in human serum

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Abstract Total serum free fatty acids (FFA_t) levels provide an important measure of the physiologic state. Most of the FFA in serum is bound to albumin; a small portion, however, is unbound. This study presents the first measurements of serum unbound free fatty acid (FFA_u) concentrations. These measurements were made possible by the development of the fluorescence probe ADIFAB (acrylodated intestinal fatty acid binding protein). In the present study ADIFAB was shown to provide the correct value of the sum of the [FFA_u] of each molecular species of long chain FFA: 1) in aqueous mixtures of FFA and ADIFAB; 2) in mixtures of FFA, human serum albumin, and ADIFAB; and 3) in serum and ADIFAB. Human serum [FFA_u] were measured in 283 samples from healthy donors and yielded a mean value of 7.5 nM with a standard deviation of 2.5 nM. This measured value is as much as 1000-fold smaller than [FFA_u] values previously estimated. The distribution of [FFA_u] values was found to be invariant with donor gender and age. Average [FFA_u] values do, as expected, exhibit a small (1.5 nM) but significant ($P < 0.001$) increase after overnight fasting. ■ FFA_u levels were found to be well correlated with total serum FFA or, equivalently, [FFA_t]/[HSA]. Given their ease and accuracy, and because [FFA_u] values increase exponentially with [FFA_t]/[HSA], [FFA_u] measurements could provide a sensitive method for assessing the physiologic state. ~Richieri, G. V., and A. M. Kleinfeld. Unbound free fatty acid levels in human serum. *J. Lipid Res.* 1995. 36: 229–240.

Supplementary key words human • serum • metabolism • ADIFAB, fluorescence

Long chain free fatty acids (FFA)² are essential for physiologic homeostasis, providing, for example, a major portion of mammalian energy needs. Because large quantities of FFA are required to meet energy needs and because long chain FFA are highly insoluble in the aqueous phase, the major portion of FFA in the blood is carried in association with albumin. A small part of the total FFA within the blood, however, dissociates from the albumin. These molecules, the unbound FFA (FFA_u), are in true aqueous solution as monomers of FFA. Serum levels of FFA_u are determined, or regulated, by the ratio of total serum FFA to total serum albumin (1, 2). Measurements of total serum FFA and human serum albumin (HSA) levels generally show that in healthy individuals, under normal physiologic conditions, the value of [FFA_t]/[HSA] is ≤ 1.0 (1, 2).

Direct measurements of serum FFA_u levels have not been done previously simply because there was no method to determine accurately the concentration of the poorly soluble long chain FFA in equilibrium with FFA binding components of serum. Recently, we have developed a fluorescent probe of FFA_u (ADIFAB), formed by derivatizing a fatty acid binding protein with the fluorescent molecule acrylodan (3). This probe specifically detects long chain FFA_u by undergoing a shift in fluorescence from 432 nm to 505 nm upon binding a single FFA_u molecule. Because the probe does not interact with other serum components and is present in small quantities, FFA_u levels can be determined simply by measuring the ratio of 505 nm to 432 nm fluorescence in an aqueous sample containing ADIFAB and FFA_u. In particular, measurements have been done of FFA_u levels for a variety of physiologic FFA in equilibrium with albumin (4).

In the present study we have developed procedures required to apply the ADIFAB methodology to measurements of serum [FFA_u]. We have used this methodology to explore the level of FFA_u in a population of healthy donors (normals) and find that FFA_u levels are narrowly distributed and are independent of population factors such as gender and age.

Abbreviations: ADIFAB, acrylodated intestinal fatty acid binding protein (I-FABP); FFA, free fatty acids; FFA_t, total FFA; FFA_u, unbound FFA; HSA, human serum albumin; NCI, National Cancer Institute; MBI, Medical Biology Institute; BCB, Biological Carcinogenesis Branch.

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²In this paper the term FFA refers to fatty acids that are not esterified. Total FFA (FFA_t) includes FFA that are bound to serum components and FFA that are unbound (FFA_u). The unbound FFA are the aqueous phase monomers of the FFA. In aqueous buffer at pH 7.4, the FFA_u monomers are primarily anions of the carboxylic acid, with admixtures of the acid and sodium salts (29). In our previous studies in which ADIFAB has been used to measure FFA_u, a different nomenclature was used (3, 4, 7). In those studies the aqueous phase monomers were referred to as FFA, not FFA_u as in the current study. The rationale for this other usage is that esterified molecules such as triglycerides do not formally contain fatty acids (carboxylic acids, salts, or anions) and therefore fatty acids are per se unesterified. We have altered the nomenclature in this paper because in previous studies total serum fatty acid levels have, for the most part, been referred to as either FFA or unesterified fatty acid.

Materials

Fatty acids and their sodium salts were purchased from Nu-chek Prep (Elysian, MN). The sodium salts were dissolved at a concentration of 20–50 mM in deionized water plus 4 mM NaOH and 25 μ M butylated hydroxytoluene and maintained under argon at -20°C as described previously (4). Essentially fatty acid-free human albumin was purchased from Sigma Chemicals (St. Louis, MO). Organic solvents were "Baker Resi-Analyzed" high purity solvents obtained from JT Baker (Phillipsburg, NJ). The buffer used in all measurements of FFA_u consisted of 10 mM HEPES, 150 mM NaCl, 5 mM KCl, and 1 mM Na_2HPO_4 , at pH 7.4. ADIFAB (acrylodan intestinal fatty acid binding protein) was prepared from acrylodan derivatized recombinant rat intestinal fatty acid binding protein (I-FABP) as described (3) and is available from Molecular Probes (Eugene, OR). ADIFAB was stored at about 60 μ M in a buffer consisting of 50 mM Tris, 1 mM EDTA, 0.5 mM PMSE, and 0.05% sodium azide, at pH 8.0.

Serum samples

Human serum samples from healthy donors were obtained from one of two repositories maintained by the National Cancer Institute (NCI) or from local volunteers (Medical Biology Institute, MBI). The NCI repositories are maintained by the Biological Carcinogenesis Branch (BCB) and by the Mayo Clinic for the Diagnosis Branch. The BCB repository contains serum samples drawn from both nominally healthy donors and patients with a variety of diseases between the years 1967 and 1974 and were stored at -70°C . The limited information available for these samples includes date of draw, age, gender, and race. The Mayo samples were collected between the years 1981 and 1987 from nominally healthy donors and patients with disease and have also been stored at -70°C . The Mayo samples were provided to us "blind" and only after completion of the FFA_u measurements was the sample code broken. Information available about these samples includes date of draw, age, gender, but not race. All samples from the NCI repositories were obtained frozen and were immediately stored at -70°C upon receipt. Serum samples from local donors (MBI) were obtained using protocols that follow NIH guidelines and these protocols were approved by the MBI institutional review board. Each donor was asked to complete a questionnaire concerning their medical history and blood from each donor in this program was HIV and Hepatitis B negative at the time of enrollment. A 10-ml sample of blood was drawn by venipuncture, generally between 8 and 12 AM; blood was allowed to coagulate, serum was removed, and either FFA_u measurements were performed immediately or the serum was frozen at -70°C .

Assays

Total serum FFA ($[\text{FFA}_t]$) was determined using the NEFA C assay (WAKO Pure Chemical Ind., Osaka, Japan). Total albumin was determined with the Albumin Reagent (BCG) Kit purchased from Sigma Diagnostics. Gas chromatography (GC) analysis of the molecular species distribution of FFA was done essentially as described previously (5). Briefly, serum was extracted by methanol-chloroform to obtain total non-esterified FFA as described by Kates (6) and margaric acid (17:0) was added as an internal standard. Thin-layer chromatography was used to separate unesterified fatty acids from other lipid components and free fatty acids eluted in ethanol from this plate were analyzed using a Hewlett-Packard 5890 series II Gas Chromatograph with a 7 m FFAP capillary column.

ADIFAB determination of FFA_u levels in mixtures of FFA

Upon binding free fatty acid, ADIFAB fluorescence shifts from 432 nm emission, in the apo form, to 505 nm with bound FFA (3). Previously we showed that $[\text{FFA}_u]$ can be determined for a single molecular species of FFA from the ratio of 505 to 432 nm fluorescence by:

$$[\text{FFA}_u] = K_d Q(R - R_o)/(R_{\max} - R) \quad \text{Eq. 1}$$

where R is the measured ratio of 505 to 432 nm intensities (with blank intensities subtracted), R_o is this ratio with no FFA_u present, R_{\max} is the value when ADIFAB is saturated, $Q = I_u(432)/I_b(432)$, $I_u(432)$ 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, with the SLM 8000C fluorometer (3), and equilibrium constants (K_d) for each molecular species of FFA were determined as described (3, 7).

As shown in the Appendix this analysis can be extended to mixtures of molecular species of FFA in which case equation 1 becomes:

$$[\text{FFA}_u] = K_d^{\text{mix}} Q(R - R_o)/(R_{\max} - R) \quad \text{Eq. 2}$$

in which $[\text{FFA}_u]$ is now the sum of the free concentrations of each of the molecular species in the mixture,

$$[\text{FFA}_u] = \sum_i [\text{FFA}_u^i] = \sum_i [\text{FFA}_u] \cdot \alpha_i = [\text{FFA}_u] \cdot \sum_i \alpha_i \quad \text{Eq. 3}$$

where the α_i are the fraction of FFA type i in the mixture and $\sum_i \alpha_i = 1$. As shown in the appendix, the effective equilibrium constant for FFA mixtures interacting with ADIFAB is,

$$K_d^{\text{mix}} = \left(\sum_i \frac{\alpha_i}{K_d^i} \right)^{-1} \quad \text{Eq. 4.}$$

in which K_d^i is the dissociation constant for the free fatty acid of type i .

To determine FFA_u levels in buffer alone, in buffer plus albumin, or in buffer plus serum, R values were measured at 37°C using an SLM 8000 C fluorometer. Measurements in buffer with or without albumin were done in glass cuvettes, while serum measurements were done in polystyrene cuvettes. Measurements without albumin or serum present were corrected for FFA binding to the walls of the cuvette, by measuring either the decrease in R value or $[^3\text{H}]\text{FFA}$ radioactivity upon transferring a mixture of FFA and ADIFAB from one cuvette to another, as described previously (3). The degree of FFA binding to the surfaces was found to be inversely proportional to the aqueous phase solubility of the FFA, amounting to between 8 and 21% of added FFA. For each sample, 10 pairs of intensities were collected at 432 and 505 nm and after subtraction of the blank intensities (sample without ADIFAB) the average and standard deviation of the R values were determined. Measured R values range from approximately 0.26 with zero FFA_u present (R_o) to a maximum of about 6.0, with standard deviation generally less than 0.4%. This corresponds to an uncertainty in the ΔR values (0.01) for low $[\text{FFA}_u]$ (7 nM oleate, for example) of ± 0.001 and therefore an uncertainty in $[\text{FFA}_u]$ of about 0.8 nM.

$[\text{FFA}_u]$ determination in complexes formed by mixtures of FFA and albumin

Previously, ADIFAB was used to determine FFA_u levels for single molecular species of FFA in the presence of albumin (4). Similar experimental procedures were used in the present study to determine $[\text{FFA}_u]$ for complexes formed with FFA mixtures and human albumin. For each mixture, FFA_u 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_o value); and 3) FFA, albumin, and ADIFAB in buffer. The same albumin (6 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 FFA addition a concentrated stock of mixtures of the sodium salts of the FFA was warmed to temperatures just above 72°C, the melting point of stearate. FFA 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 pipette. Between each FFA 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.

R values measured with ADIFAB alone or in the

presence of membranes, cells, or a variety of proteins are constant to within 0.5% over times longer than 5 h (refs. 3, 7, and data not shown). In the presence of albumin or serum, however, a very slow increase in R value of about 0.01 per h is observed and corresponds, at low $[\text{FFA}_u]$, to an apparent increase of about 5 nM/h (for oleate). This effect is eliminated by the procedure described above where parallel R_o measurements (sample 2) are made at the same time as those with FFA (sample 3), as the same time-dependent increase in R occurs in the absence or presence of FFA (data not shown).

$[\text{FFA}_u]$ determination in serum

Determination of $[\text{FFA}_u]$ in serum samples was done using essentially the same methods as for albumin. Serum samples (fresh or thawed from -70°C) were prepared by a 100-fold dilution of 100% serum in buffer (15 μl of 100% serum added to 1.5 ml buffer), yielding albumin concentrations of about 6 μM , about the same as used in the HSA measurements. Diluting serum 100-fold does not affect $[\text{FFA}_u]$ because FFA_u/HSA complexes are able to buffer FFA_u levels, so long as $[\text{HSA}]$ exceeds 0.5 μM (4). In fact, virtually identical FFA_u levels were found using 10-fold higher (60 μM) serum concentrations (data not shown). As discussed previously, ADIFAB itself has little effect on $[\text{FFA}_u]$ in these measurements because of the large FFA binding capacity of albumin and because HSA and FFA are present at much greater concentrations than ADIFAB. For each measurement, two samples were prepared from a given donor consisting of a 1% serum blank and 1% serum plus 0.5 μM ADIFAB. A third sample was also used, consisting of a solution of 6 μM fatty acid-free human albumin plus 0.5 μM ADIFAB (R_o) and an albumin blank. For these samples the background (serum blank) contributions to fluorescence were less than 0.5% of the ADIFAB intensities. Diluted serum samples were used in these measurements both to limit the amount of ADIFAB required, while maintaining the background fluorescence at the 0.5% level, and to limit the amount of serum required for each FFA_u determination. Each of these samples were incubated at 37°C for similar times and R value measurements of serum and HSA were done within 2 min of each other in order to compensate for the slow R value increases described above.

In a mixture of FFA, the sum of the unbound concentrations is determined by the ADIFAB fluorescence (equations 2 and 3). To obtain $[\text{FFA}_u]$ from the measured R value, the average K_d^i weighted by the fraction of each of the unbound molecular species of FFA must be known. The interaction of a single molecular species of FFA and multiple albumin binding sites is well described by a step-wise equilibrium analysis and this analysis can be used to determine the unbound concentration of each of the single FFA in equilibrium with albumin (4). In this previous

analysis the stepwise model was parameterized so as to reproduce the observed FFA_u levels in equilibrium with albumin and therefore the model's predictions of FFA_u are independent of whether it correctly describes the actual binding mechanism. In the present study we have calculated the unbound concentration of each FFA in mixtures of FFA in equilibrium with albumin or in serum by assuming that the previous stepwise analysis applies independently to each FFA in the mixture. These calculated $[\text{FFA}_u^i]$ for each FFA were used to evaluate the corresponding set of α_i

$$\alpha_i = \frac{[\text{FFA}_u^i]}{\sum_i [\text{FFA}_u^i]}$$

and these values in turn were used to calculate K_d^{mix} according to equation 4. In this study the measured $[\text{FFA}_u]$ values, in equilibrium with serum or with albumin, were determined using these K_d^{mix} values together with the measured R values and equation 2.

ADIFAB selectivity for long chain FFA

The ADIFAB response is specific for long chain FFA (3). K_d values of the different FFA primarily reflect aqueous solubility differences and for this reason ADIFAB is less selective for shorter chain FFA (Table 1). In order to gauge whether certain other molecules that might be present in serum could interfere with the FFA_u determinations, the ADIFAB response was measured with the unbound concentrations of these molecules shown in Table 1. As seen, these molecules either have no effect or have such low affinities for ADIFAB that, as most of these molecules are hydrophobic, their effect is probably negligible at physiologic free concentrations.

RESULTS

Response of ADIFAB to FFA_u mixtures

Previously it was shown that binding of a single molecular species of FFA to ADIFAB is well characterized by equation 1. This equation relates the concentration of the FFA_u of a particular FFA to the ratio of ADIFAB fluorescence intensities at 505 and 432 nm (3). To determine $[\text{FFA}_u]$ in mixtures of different FFA it is necessary to show that ADIFAB responds to mixtures of FFA as predicted by equation 2. For this purpose we examined the binding to ADIFAB of a binary mixture of oleate-arachidonate 1:1 and a quaternary mixture of palmitate-stearate-oleate-linoleate 27:11:41:21. We chose these mixtures in the first case because oleate and arachidonate have a nearly 6-fold difference in K_d values (Table 1) and in the second case because the quaternary mixture

TABLE 1. The ADIFAB response: effects of potential interferants^a

Molecule ^b	Concentration ^c		K_d^d
	μM	μM	
Stearate ^e			0.08
Oleate ^f			0.28
Palmitate ^f			0.31
Linoleate ^f			0.97
Arachidonate ^f			1.63
Linolenate ^f			2.50
Myristate ^e			3.4
Palmitoleate ^e			2.7
Cholate ^e			320
Deoxycholate ^e			50
Chenodeoxycholate ^e			28
Monolein ^e			190
Amino acids ^h	>1000		0
L-Thyroxine	5		0
Lysocleithin	100		0
Sphingosine	50		0
Leukotriene C4	30		0
Prostaglandin D2	6		0
5-HETE	3		0
15-HETE	3		0
Bilirubin	10		0
Cholesterol	10		0
Triolein	100		0
Docosanol	10		0

^aThe response of ADIFAB was determined both as a change in R value upon adding a given molecule to apo-ADIFAB and, if no R value change occurred, as an effect on the R value of oleate-bound ADIFAB.

^bAlthough FFA were added as their sodium salts, many of the other molecules were added from solutions in ethanol. Ethanol itself increases the ADIFAB R value and therefore the ethanol concentration was limited to 0.1% by volume, and corrections were made for the ethanol contribution to the R value changes.

^cThe R value was monitored as a function of the concentration of molecule added up to the maximum concentration listed in this column for those molecules that exhibited no effect on R.

^d K_d values were determined as described previously (3) for those molecules that elicited an increase in R value.

^eAnel, Richieri, and Kleinfeld (7).

^fRichieri, Ogata, and Kleinfeld (3).

^gG. V. Richieri and A. M. Kleinfeld, data not shown.

^hTryptophan at 180 μM and tyrosine at 900 μM reduced the R value of oleate-bound ADIFAB by 10%.

represents about 90% of the FFA composition of serum. For each mixture the ADIFAB fluorescence ratio (R) was measured as a function of the total concentration of these mixtures. Measured $[\text{FFA}_u]$ values were calculated from the measured R values using equations 2 and 4 and the results are shown in Fig. 1. The quantity Added $[\text{FFA}_t]$ plotted along the abscissa is the actual concentration of free fatty acid, the total FFA added to the cuvette corrected for wall binding. The results show that Measured $[\text{FFA}_u]$ is linearly related to Added $[\text{FFA}_t]$ with a slope of unity for both mixtures, indicating that the $[\text{FFA}_u]$ determined from the ADIFAB response and equation 2, is equal to the actual $[\text{FFA}_t]$ present in solution.

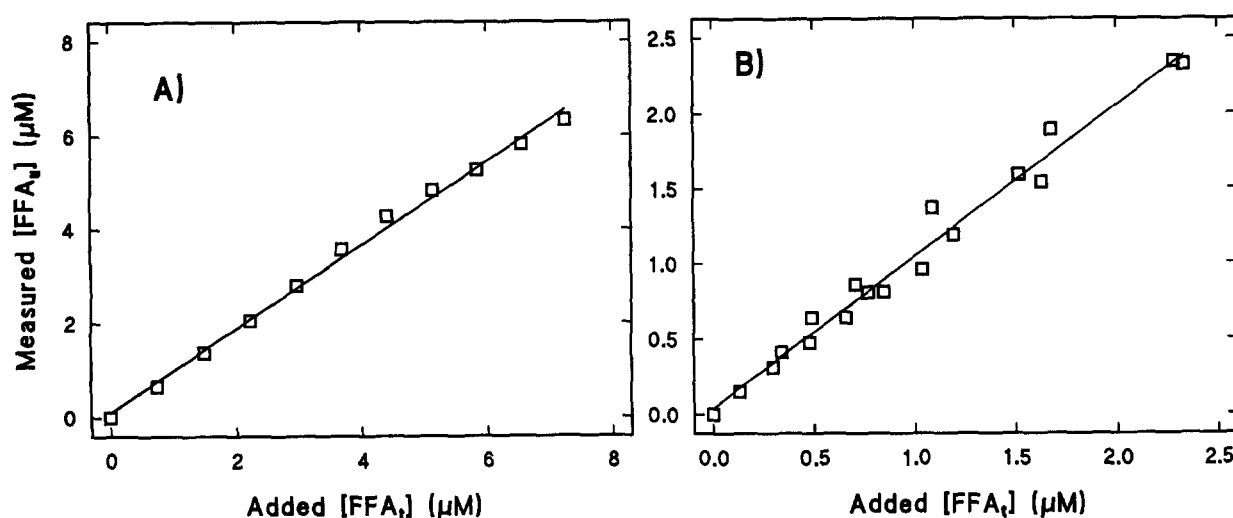


Fig. 1. ADIFAB response to mixtures of FFA_u . The values of Measured $[\text{FFA}_u]$ on the ordinate were calculated from the ADIFAB fluorescence intensities at 505 and 432 nm (R) and equation 2. The value of K_d^{mix} in equation 2 was calculated using equation 4, the individual K_d^{mix} values of Table 1, and the FFA mole fractions corresponding to each of the mixtures. A) Binary mixtures of oleate-arachidonate 1:1 were added at the indicated concentrations and the measured R value was used to obtain the Measured $[\text{FFA}_u]$ using equation 2 with K_d^{mix} equal to $0.48 \mu\text{M}$. The slope of the linear fit was 0.89 ± 0.02 . B) Quaternary mixtures of stearate-palmitate-oleate-linoleate 11:27:41:21 were used, with a K_d^{mix} value $0.34 \mu\text{M}$ to obtain the Measured $[\text{FFA}_u]$. The slope of the fit was 1.00 ± 0.04 . In both A) and B) $0.5 \mu\text{M}$ ADIFAB was used and the fraction of added FFA binding to the cuvette walls was determined as described previously (3). All measurements were performed in triplicate.

Response of ADIFAB to FFA mixtures in equilibrium with albumin

Although binding of FFA to cell membranes and lipoproteins competes with binding to albumin in vivo (in the circulation) at high $[\text{FFA}_t]/[\text{HSA}]$ values (8), the extent of FFA binding to albumin in serum is expected to greatly exceed that of FFA binding to all other serum components (1, 2). In this event, the interaction between FFA and albumin and, therefore, the ratio of total serum FFA to total albumin, $[\text{FFA}_t]/[\text{HSA}]$, should determine serum levels of $[\text{FFA}_u]$ (1, 4). For equivalent $[\text{FFA}_t]/[\text{HSA}]$ values, ADIFAB should measure identical $[\text{FFA}_u]$ values in serum and albumin solutions. Thus,

comparison of the ADIFAB response in albumin and serum should provide a stringent test for the validation of the method in serum. Measurements of $[\text{FFA}_u]$ in FFA_t/HSA complexes were made using free fatty acids mixtures in the compositions: stearate-linoleate 1:1, palmitate-stearate-oleate-linoleate 27:11:41:21, and palmitate-stearate-oleate-linoleate-arachidonate-linolenate 25:10:38:22:3:2. These mixtures were chosen a) because linolenate and stearate possess, respectively, the lowest and highest binding affinities for both ADIFAB and albumin; b) because the quaternary mixture represents about 90% of the serum distribution; and c) because the mixture of six FFA is virtually identical to the serum distribution (see Table 4).

Because the binding affinities of FFA to HSA are differ-

TABLE 2. Estimated $[\text{FFA}_u]$ and K_d^{mix} values in FFA_u mixtures

Mixture ^a	PA	SA	OA	LA	AA	LNA	$[\text{FFA}_u]$	K_d^{mix}
							nM	μM
Binary		0.7				6.9	7.6	0.70
Quaternary	1.6	0.2	2.7	1.5			6.0	0.34
Six FA	1.5	0.14	2.6	1.5	2.0	0.2	8.0	0.44

All values were evaluated for $[\text{FFA}_t]/[\text{HSA}] = 1$. The individual $[\text{FFA}_u]$ values were determined using the association constants for each FFA in equilibrium with albumin that were obtained from a stepwise equilibrium analysis (4). The sum of these individual values, $[\text{FFA}_u]$, is indicated and K_d^{mix} was calculated according to equation 4 using the individual FFA_u values to determine α_i as described for equation 3. $[\text{FFA}_u]$ values are in nM and K_d^{mix} values are in μM . The K_d^{mix} values are independent of $[\text{FFA}_t]/[\text{HSA}]$ for the quaternary and mixtures with six FA, but increase slightly for the binary mixture, from 0.63 ($[\text{FFA}_t]/[\text{HSA}] = 0.5$) to 0.83 ($[\text{FFA}_t]/[\text{HSA}] \geq 3.0$) with an average value of 0.78 for $[\text{FFA}_t]/[\text{HSA}] \leq 3.0$.

^aThe proportions of total FFA of each molecular species in the mixtures were: binary, SA-LNA 1:1; quaternary, PA-SA-OA-LA 27:11:41:21; and the values for the six FFA mixture are the same as the serum values in Table 4: PA-SA-OA-LA-AA-LNA 25:10:38:22:3:2. Abbreviations: PA, palmitate; SA, stearate; OA, oleate; LA, linoleate; AA, arachidonate; LNA, linolenate.

ent for each molecular species of FFA, the distribution of $[FFA_u]$ in equilibrium with HSA differs from the molecular species distribution of total FFA. To evaluate $[FFA_u]$ in mixtures of FFA in equilibrium with HSA, the distribution of $[FFA_u]$ must be estimated so that K_d^{mix} can be calculated from equation 4. As described in Methods, the $[FFA_u]$ distribution was estimated using values for the unbound level of each FFA obtained previously (4) from the stepwise binding results for individual FFA. The K_d^{mix} values resulting from this evaluation for the above FFA mixtures are shown in Table 2. Figure 2 shows the $[FFA_u]$ determined from the measured R and the above K_d^{mix} values; along with $[FFA_u]$ predicted by the individual stepwise binding model, as a function of the ratio of $[FFA_t]/[HSA]$. As is seen, both the measured and predicted values are in good agreement for FFA_t/albumin ratios ≤ 2 , the normal physiologic range. At higher ratios $[FFA_u]$ are considerably greater than predicted. These results also show, just as those for single FFA (1, 4), that at FFA_t/HSA values above two, FFA_u levels increase exponentially.

Serum levels of FFA_u

To evaluate $[FFA_u]$ from R values measured in serum, a value for K_d^{mix} of $0.44 \mu M$ was used, as predicted from the consensus distribution of FFA_t in serum (see Table 4). Serum FFA_u levels were determined in 283 serum samples of nominally healthy donors obtained either from the NCI's BCB or Mayo repositories ($n = 151$) or collected locally and designated MBI ($n = 132$) (Fig. 3). The mean value of the distribution of all samples is 7.5 nM with a standard deviation of 2.5 nM (Fig. 3A). Because samples from the NCI repositories were stored for between 10 and 30 years, and because no information is available concerning the fasting state and little about the medical history of the donors, the $[FFA_u]$ distribution of each of the NCI and MBI populations was also analyzed separately. As is seen in Fig. 3B and C and in Table 3, the mean NCI ($7.6 \pm 2.6 \text{ nM}$) and MBI ($7.5 \pm 2.4 \text{ nM}$) values are virtually identical.

Subpopulations of these distributions have been examined to determine whether gender, age, or fasting affects

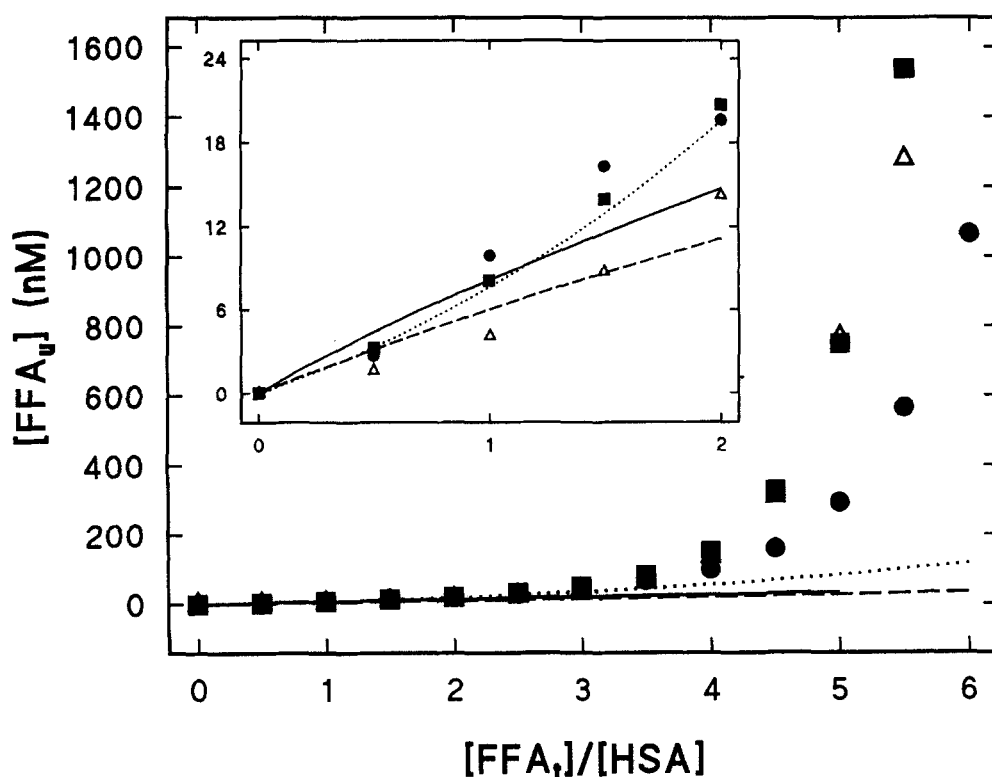


Fig. 2. $[FFA_u]$ levels in mixtures of FFA with HSA. $[FFA_u]$ values were determined, from the ADIFAB R value, in binary (●, stearate-linolenate 1:1); quaternary (△, palmitate-stearate-oleate-linolenate 27:11:41:21); and the six FFA (■, palmitate-stearate-oleate-linolenate-arachidonate-linolenate 25:10:38:22:3:2) mixtures of FFA in equilibrium with $6 \mu M$ HSA. The dotted, dashed, and solid lines represent the $[FFA_u]$ calculated by summing $[FFA_u]$ values for each FFA obtained from the stepwise equilibrium analysis (4) for the binary, quaternary, and six FFA mixtures, respectively.

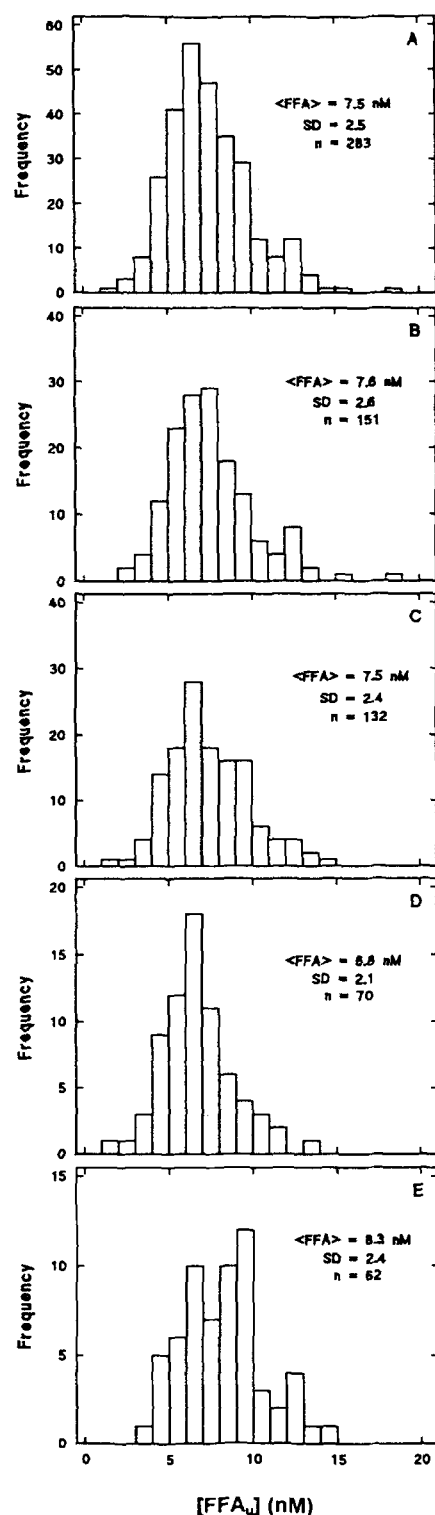


Fig. 3. Population distribution of $[FFA_u]$ in normal human serum. $[FFA_u]$ values were determined from the measured ADIFAB R values with K_d^{mix} equal to $0.44 \mu M$. Serum samples were obtained, as explained in the text from NCI repositories of healthy donors and from samples collected locally (MBI). A) All normals; B) NCI only; C) MBI only; D) MBI nonfasting; E) MBI fasting. The average values and standard deviations and the number of samples in each of these populations are shown in each figure and in Table 3.

TABLE 3. $[FFA_u]$ values in human serum from healthy donors

Group	FFA Values		
	All	NCI	MBI
	<i>nM</i>		
All	7.5 ± 2.5 (283)	7.6 ± 2.6 (151)	7.5 ± 2.4 (132)
Male	7.6 ± 2.6 (174)	8.0 ± 2.8 (73)	7.4 ± 2.4 (100)
Female	7.3 ± 2.3 (109)	7.2 ± 2.2 (78)	7.7 ± 2.4 (32)
Fasting ^a			8.3 ± 2.4 (62)
Nonfasting			6.8 ± 2.1 (70)

$[FFA_u]$ values are in nM and were determined using equation 2 with $K_d^{mix} = 0.44 \mu M$. Values shown are population averages, standard deviations, and, in parentheses, the number of samples in the given population.

^aSamples from fasting MBI donors were obtained after an overnight fast (12–16 h). A Student's *t* test yielded a probability of < 0.0001 that fasting and nonfasting means were equal.

the distribution of $[FFA_u]$ values. As shown in Table 3 the male–female difference in population averages is not significant ($P < 0.51$). Age-grouped $[FFA_u]$ levels were measured with samples pooled from both the NCI and MBI populations in 10-year intervals (data not shown). The results of these measurements were consistent, at the 95% confidence limits, with a slope of zero (0.01 ± 0.02) or with a constant $[FFA_u]$ value of (7.1 nM). In contrast to the lack of dependence on gender or age, the increase of 1.5 nM in the mean $[FFA_u]$ level in donors who fasted for 12–16 h before the serum sample was drawn is highly significant ($P < 0.0001$).³

The reproducibility of the $[FFA_u]$ measurements in identical samples was determined from an NCI/Mayo repository set of 26 sample pairs that were included blind in a panel of 316 samples (from both healthy donors and patients with disease). Reproducibility was also studied in repeated measurements performed on 96 MBI samples in which each sample was measured an average of more than 3 times. The average differences found in the NCI/Mayo and MBI sets of 1.1 ± 1.0 nM and 1.0 ± 0.8 nM, respectively, are consistent with the intrinsic measurement uncertainty of approximately 0.8 nM (Methods). This level of reproducibility of the measurement is consistent with the level of variation of $[FFA_u]$ levels in an individual donor. Twenty eight of the MBI donors contributed an

³Although the increase in $[FFA_u]$ with fasting is consistent with increases in FFA, observed previously (for example, ref. 30) it is possible, in principle, that the approximately 30% increases in $[FFA_u]$ observed here are due to alterations in FFA distributions in the fasting individuals that would result in an approximately 30% increased K_d^{mix} . Measurements were not done in the present study to compare FFA distributions in the fasting and non-fasting populations, because the expected changes would probably be beyond the accuracy of the GC method used here. Distributions measured by others in fasting individuals are consistent with no change and these studies also found virtually no change in FFA distributions in patients with diabetes (10).

average of 3 (minimum of 2 and maximum of 6) samples on separate occasions. The average standard deviation of $[FFA_u]$ values of the individual donors was 1.3 nM, also consistent with the intrinsic measurement uncertainty.

Although agreement between NCI and MBI values is consistent with no sample degradation after long term storage, several additional measurements were done to address directly the possibility of sample degradation. Measurements from the set of the 96 MBI samples, described above, in which the first measurement was done with a fresh sample and subsequent measurements involved repeated freeze (-70°C)/thaw cycles of the same sample over a period of 20 days, failed to exhibit any significant change in $[FFA_u]$. In addition, 22 MBI samples that had been measured when fresh, were remeasured after storage at -70°C for between 1 and 1.5 years. The average $[FFA_u]$ values found in this study were 7.1 ± 2.7 nM and 7.1 ± 2.3 nM in the fresh and stored samples, respectively, and the average difference of individual values 0.7 ± 0.5 was also consistent with the uncertainty of the measurement itself (± 0.7 nM). Total FFA measurements were also consistent with no effect of long term storage at -70°C . Average total $[FFA_t]$ from 16 fresh serum samples was 502 ± 150 μM as compared with a value of 490 ± 160 μM from 8 samples that had been stored for 1.5 years at -70°C .

Comparison of $[FFA_u]$ levels in serum and $[FFA_t]$ /albumin mixtures

To compare $[FFA_u]$ values measured in serum with those in $[FFA_t]$ /albumin mixtures, measurements were done in serum samples to determine the total FFA concentration, the distribution of FFA molecular species, and the concentration of albumin. Samples used in these measurements were those whose individual measured $[FFA_u]$ values corresponded approximately to the popula-

tion mean. The average total $[FFA_t]$ and $[HSA]$ values measured in 16 MBI samples were found to be 502 ± 150 and 680 ± 35 μM , respectively, corresponding to an average $[FFA_t]/[HSA]$ value of 0.74. The average value of $[FFA_u]$ from these 16 samples was 7.2 ± 1.0 nM. These results are in good agreement with previous determinations of total serum $[FFA_t]$ and $[albumin]$ (2, 9). Analyses of the molecular species distribution from 12 MBI samples were averaged and the results (Table 4) are in reasonable agreement with previous determinations (10, 11). Table 4 also shows that substantial variations in these values have only a modest effect on the predicted K_d^{mix} values and therefore even substantial alterations of the FFA molecular species distribution would alter $[FFA_u]$ by less than about 14%.

The values found for the mean $[FFA_u]$ and the corresponding $[FFA_t]/[HSA]$ in this set of serum samples, 7.2 nM and 0.74, respectively, can be compared with values obtained with the mixture of six FFA and HSA designed to reproduce the FFA composition of serum. Interpolation of the results in Fig. 2 shows that the $[FFA_u]$ value corresponding to $[FFA_t]/[HSA]$ of 0.75 for the mixture of six FFA is about 6.5 nM, in good agreement with the serum value. Further evidence that the ADIFAB measurements in serum correctly determine $[FFA_u]$ levels and are well correlated with $[FFA_t]/[HSA]$ were obtained by titrating a serum sample with HSA or the mixture of six FFA (Fig. 4). As seen in Fig. 4A, the concentration of serum $[FFA_u]$ either decreases upon addition of fatty acid-free HSA or increases upon addition of FFA. These changes in $[FFA_u]$ with $[FFA_t]/[HSA]$ are in good agreement with the results obtained in mixtures of FFA and albumin (Fig. 2). Moreover, as seen in Fig. 4B, the increase in $[FFA_u]$ observed upon titrating a serum sample with increasing concentrations of the six-FFA mixture (Fig. 4A) agrees well with the increase in $[FFA_u]$ measured in in-

TABLE 4. Molecular species distribution of total human serum fatty acids

Source	Palmitate	Stearate	Oleate	Linoleate	Arachidonate	Linolenate	K_d^{mix}
	% of total						μM
This study ^a	21 \pm 6	9 \pm 3	41 \pm 6	24 \pm 5	3 \pm 1	2 \pm 1	0.45
Miwa and Yamamoto ^b	27 \pm 4	8 \pm 2	32 \pm 3	26 \pm 3	4 \pm 1	3 \pm 1	0.49
Moilanen ^c	21 \pm 2	14 \pm 4	41 \pm 1	16 \pm 4	2 \pm 1	1 \pm 1	0.38
Average ^d	25	10	38	22	3	2	0.44

$[FFA_t]$ values, as a percent of the total, were determined by GC or HPLC in three separate studies. K_d^{mix} was calculated for $[FFA_t]/[HSA] = 1$ using the same procedure as described for Table 2. Although other molecule species were resolved in all studies, primarily myristate (3%) and palmitoleate (3.5%), K_d have not been determined accurately for these FFA and therefore only the six FFA shown were used to estimate K_d^{mix} (in μM). The contributions of these other FFA were subsumed into the linoleate and arachidonate contributions and all values were adjusted so the sum was 100%. As a consequence, the values shown are slightly altered compared to the originals.

^a $[FFA_t]$ values were determined by GC analysis of serum of 12 healthy donors. Values shown are averages and standard deviations of the 12 samples given as a percent of the total serum FFA.

^bReference 10.

^cReference 11.

^dAverages represent simple averages of the three studies.

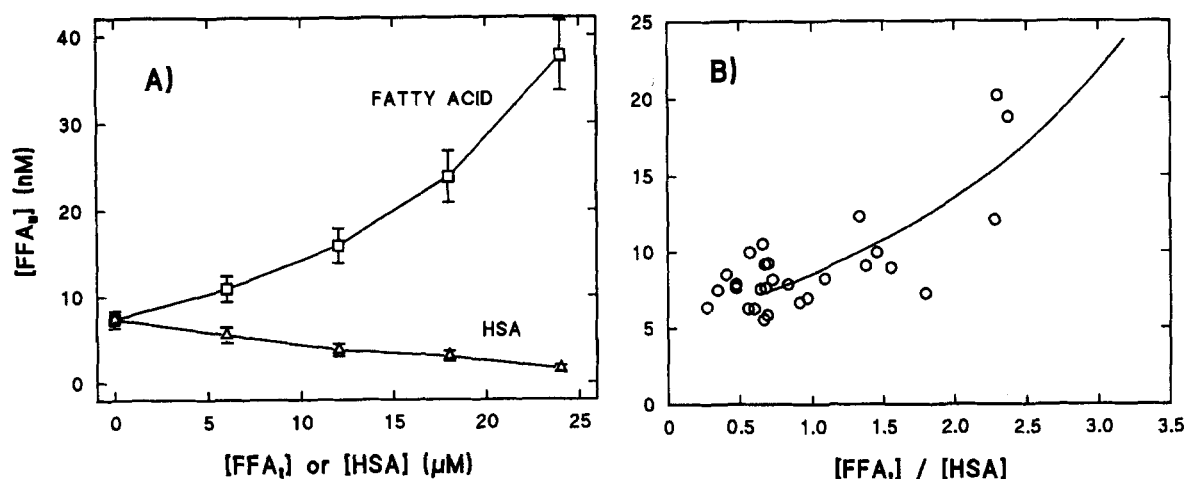


Fig. 4. Variation of FFA_u levels in serum are well correlated with $[FFA_t]/[HSA]$. A) A 1% sample of human serum titrated with either increasing concentrations of the six FFA mixture or HSA. B) Measurements of $[FFA_t]$ (corresponding to 100% serum) and $[FFA_u]$ in 28 samples from the MBI population. The solid curve is a smoothed version of the line connecting the data points in the upper part of panel A and is shown to demonstrate that the observed variation in FFA_u levels that occurs naturally in serum agrees well with that observed upon adding FFA.

dividual serum samples taken from donors whose measured total FFA concentrations increase proportionately. The response of ADIFAB in serum is, therefore, virtually indistinguishable from its response in FFA-albumin mixtures. Thus in both model and physiologic systems $[FFA_u]$ measured by ADIFAB is well correlated with $[FFA_t]/[HSA]$, strongly suggesting that such measurements are not affected significantly by interference with other serum components and do indeed reflect $[FFA_u]$ levels.

DISCUSSION

The results of this study are the first to provide measurements of serum levels of FFA_u . Measurements of 283 samples from healthy donors show that the distribution of $[FFA_u]$ values is normal with an average of 7.5 nM and a standard deviation of 2.5 nM. $[FFA_u]$ values from the total donor population were found to be invariant with donor gender and age, but increase significantly in fasting, consistent with physiologic homeostasis.

Previously, FFA_u levels expected in serum have been estimated from FFA-albumin equilibrium constants or in one case by partition between water and albumin containing red cell ghosts, and these estimates range between 5- and 1000-fold greater than the $[FFA_u]$ values measured in the present study (12–15). These overestimates for $[FFA_u]$ primarily reflect the use of FFA-albumin association constants that were too small (4). Although no previous measurements of serum $[FFA_u]$ have been reported, the values observed in this study are in excellent agreement with measurements of FFA_u levels buffered by human albumin. The ratio of FFA_t to albumin measured in this study (0.74), for a subset of MBI samples correspond-

ing to the mean $[FFA_u]$ (7.2 nM), is in good agreement with the ratio reported previously for serum of healthy (normal) donors (2, 9). For this ratio, the measured $[FFA_u]$ value is about 6.5 nM in HSA and FFA mixtures that simulate the serum distribution (Fig. 2). In addition, $[FFA_u]$ values were also calculated for equilibrium binding of FFA mixtures to human albumin, assuming no competition among the FFA, the serum $[FFA_t]$ distribution of Table 4, and the individual binding constants for each FFA obtained from a stepwise equilibrium model (4). The value of $[FFA_u]$ calculated in this way for a $[FFA_t]/[HSA]$ ratio of 0.75 was also 6.5 nM, in good agreement with the value measured in serum.

It is apparent from Fig. 2 that the measured $[FFA_u]$ values greatly exceed those calculated, assuming no competition among the FFA, for $[FFA_t]/[HSA]$ values exceeding 2.0 (see the legend of Table 2 for this calculation with $[FFA_t]/[HSA] = 1$). Because the $[FFA_u]$ for single FFA are accurately predicted by this calculation for $[FFA_t]/[HSA]$ values as high as 5, we suggest that the deviation shown in Fig. 2 reflects binding competition among the FFA molecular species that is apparent once a significant fraction of albumin binding sites are occupied. Indeed, a much improved agreement with the measured $[FFA_u]$ at $[FFA_t]/[HSA]$ values exceeding 3.0 is obtained by extending the single ligand stepwise procedure to multiple ligands (G. V. Richieri and A. M. Kleinfeld, results not shown). This theory, however, overestimates competition at low $[FFA_t]/[HSA]$ values, indicating that a stepwise model cannot provide a completely adequate description of multiple FFA binding to albumin. Thus, an accurate determination of $[FFA_u]$ in serum with $[FFA_t]/[HSA]$ values greater than 2, as occurs in specific disease states (see below), will require a more detailed un-

derstanding of the interaction of FFA and albumin. Even in these cases, however, preliminary studies indicate that the increase in $[FFA_t]/[HSA]$ values observed in these disease states is well correlated with increased values of $[FFA_u]$. The accuracy of the FFA_u measurements might also be compromised because binding of several ligands, other than FFA, with albumin has been found to decrease with increasing albumin concentration (16) and most of the measurements in the present study were done using serum samples diluted 100-fold. The binding of FFA to albumin, however, is not dependent on albumin concentration because: 1) identical FFA_u values were obtained with serum samples diluted only 10-fold (Methods), and 2) FFA_u for oleate binding to albumin was found to be independent of albumin concentrations between 5 and 500 μM (data not shown).

As discussed in Methods, a variety of potential interferants that might be present in serum were found to have little or no effect on the ADIFAB response in aqueous solution, consistent with the expected specificity for fatty acids of the intestinal fatty acid binding protein (ref. 3 and Table 1). Moreover, the agreement between $[FFA_u]$ values found in serum and solutions of FFA mixtures and human albumin, for the same $[FFA_t]/[HSA]$ values, provides assurance that serum interferants do not significantly affect $[FFA_u]$ measurements and is also consistent with albumin being the primary buffer of serum FFA_u levels. Finally, directly increasing total serum FFA or $[HSA]$, respectively increases and decreases FFA_u levels (Fig. 4), providing additional evidence that the quantity measured by ADIFAB is indeed serum $[FFA_u]$. However, one serum component, hemoglobin, has been found to be a potential source of interference. By selectively absorbing fluorescence at 432 nm, hemoglobin, which does not bind to ADIFAB, causes the 505/432 ratio to increase. Although this contribution can be accounted for by applying an inner filter correction, we have, in the present study, excluded all samples possessing any visible red tint ($OD(412) > 0.01$).

Serum FFA (and albumin) levels have been used to gauge physiologic homeostasis (2, 9). Total serum FFA measurements cannot, however, provide the same measure of homeostasis as the direct FFA_u determination, primarily because $[FFA_u]$ values increase exponentially with $[FFA_t]/[HSA]$. For example, results from measurements of FFA-HSA equilibria predict that a 4-fold increase in $[FFA_t]/[HSA]$, from 1 to 4, corresponds to a 19-fold change in $[FFA_u]$, from 8 to 151 nM (Fig. 2). In addition, measurements of total FFA either by extraction or enzymatic methods are relatively inaccurate and lengthy (refs. 10, 17–20, and the Wako kit used here). In contrast, determination of $[FFA_u]$ using ADIFAB is accurate and involves only the single step of adding ADIFAB to the serum sample. The precision of the measurement, based on multiple measurements of the same se-

rum samples, is about 1 nM and the accuracy of this value, based on comparison with FFA mixtures with albumin and calculation, is also about 1 nM.

If FFA_u levels are well regulated in healthy subjects, deviations from these values might provide an important indicator of the physiologic state. Because $[FFA_u]$ are functions of the FFA_t /albumin ratios, $[FFA_u]$ might increase because $[FFA_t]$ increases or $[albumin]$ decreases, or both. Previous studies of FFA_t /albumin do, in fact, indicate that $[FFA_t]$ and/or this ratio increase in a variety of pathologies including, diabetes (10, 21), cancer (22, 23), and sepsis (24). $[FFA_u]$ should be especially sensitive to deviations from homeostasis because, in interactions with serum albumin, $[FFA_u]$ values increase exponentially with the ratio of total FFA to albumin. Preliminary results from studies in progress are, in fact, consistent with elevations of FFA_u levels that may greatly exceed the average value obtained for healthy donors. For example, in patients with advanced cancer, $[FFA_u]$ values as high as 35 nM have been observed (G. V. Richieri et al., private communication) and, in patients undergoing angioplasty, values that exceed 100 nM have been observed (R. Davis et al., private communication). Although these values were estimated using the K_d^{mix} (0.44 μM) appropriate for $[FFA_t]/[HSA]$ values ≤ 2 and as discussed above may not be accurate, the observed large increases were well correlated with large increases in $[FFA_t]$.

Elevated FFA_u levels may not only be important indicators of pathophysiology but, based upon in vitro studies, may themselves be deleterious (8, 25–27). Because FFA_u levels in vivo (within circulating whole blood) cannot be measured directly, it is essential to know how the measured serum FFA_u levels are related to in vivo FFA_u levels. As far as FFA are concerned, the circulation and serum differ because: 1) in the circulation, FFA levels are steady state values (FFA_t turnover is complete within 1–3 min) whereas, in serum, equilibrium applies; and 2) in the circulation, FFA binding to cell surfaces and lipoproteins compete with albumin, especially at high $[FFA_t]/[HSA]$ values (8), whereas, in serum, binding to albumin dominates. The serum $[FFA_u]$ values observed in this study are consistent⁴ with observed turnover rates of FFA_t in the circulation, suggesting that the steady state

⁴A rough estimate of the steady state levels in the circulation can be obtained as follows. We assume that the turnover of FFA in the circulation occurs by the dissociation of FFA from albumin, followed by diffusion of FFA through the aqueous phase, and finally capture of the FFA by capillary endothelium. Using a FFA diffusion coefficient of 1×10^{-5} cm²/s and a capillary diameter of 5 μm , the half time for clearance of FFA from the aqueous phase by diffusion is about 7×10^{-4} s. In this amount of time, using a dissociation rate constant of FFA from albumin of 0.04 s⁻¹ (31), about 2.8×10^{-5} of the FFA bound to albumin will have dissociated. Thus for FFA_t /albumin equal to 0.74, and an albumin concentration of 600 μM , the amount of FFA that dissociates from albumin in the time required for clearance is about 14 nM, in reasonable agreement with the 7 nM values measured in this study.

conditions in the circulation can be approximated by the equilibrium values in serum. Because equilibrium between FFA_u and albumin, and FFA_u and the other FFA binding components within the circulation, is likely maintained upon separating serum from whole blood, the FFA_u values observed in serum should be virtually identical to those in the circulation. Thus serum FFA_u levels should provide an accurate estimate of FFA_u values within the circulation even at the high $[\text{FFA}_t]/[\text{HSA}]$ values that may be associated with certain disease states. ■

APPENDIX

Equation 2 can be derived by extension of the same approach used for equation 1 and described by Gryniewicz, Poenie, and Tsien (28) to express free calcium levels in terms of fluorescence intensity ratios. Accordingly, the fluorescence intensity at any emission wavelength is composed of parts corresponding to bound and unbound ADIFAB

$$I(\lambda) = [\text{ADIFAB}_b] I_b(\lambda) + [\text{ADIFAB}_u] I_u(\lambda) \quad \text{Eq. A1}$$

in which $[\text{ADIFAB}_b]$ and $[\text{ADIFAB}_u]$ are the bound and unbound concentrations of ADIFAB and $I_b(\lambda)$ and $I_u(\lambda)$ are the specific fluorescence intensities of these components at emission wavelength λ . The ratio of intensities at two wavelengths in which 1 corresponds to 505 and 2 to 432 nm is:

$$R = \frac{[\text{ADIFAB}_b] \cdot I_b(1) + [\text{ADIFAB}_u] \cdot I_u(1)}{[\text{ADIFAB}_b] \cdot I_b(2) + [\text{ADIFAB}_u] \cdot I_u(2)} \quad \text{Eq. A2}$$

For each FFA of molecular species i , the following equilibria apply:



and therefore

$$[\text{ADIFAB}_b^i] = \frac{[\text{ADIFAB}_u] \cdot [\text{FFA}_u^i]}{K_d^i} \quad \text{Eq. A4}$$

The total concentration of ADIFAB bound to all FFA in the mixture is:

$$\Sigma_i [\text{ADIFAB}_b^i] = \Sigma_i \frac{[\text{ADIFAB}_u] \cdot [\text{FFA}_u^i]}{K_d^i} = [\text{ADIFAB}_u] \cdot \Sigma_i \frac{[\text{FFA}_u^i]}{K_d^i} \quad \text{Eq. A5}$$

Furthermore α_i can be expressed as:

$$[\text{FFA}_u] = \Sigma_i [\text{FFA}_u^i] = \Sigma_i [\text{FFA}_u] \cdot \alpha_i = [\text{FFA}_u] \cdot \Sigma_i \alpha_i \quad \text{Eq. A6}$$

and by substituting equation A6 into equation A5

$$\Sigma_i [\text{ADIFAB}_b^i] = [\text{ADIFAB}_u] \cdot [\text{FFA}_u] \cdot \Sigma_i \frac{\alpha_i}{K_d^i} = [\text{ADIFAB}_u] \cdot [\text{FFA}_u] \cdot (K_d^{\text{mix}})^{-1} \quad \text{Eq. A7}$$

Substitution of equation A7 for $[\text{ADIFAB}_b]$ in equation A2 and solving the resulting expression for $[\text{FFA}_u]$ yields equation 2.

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