Quantitative analysis of uptake of free fatty acid by mammalian cells: lauric acid and human erythrocytes

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Abstract Quantitative aspects of the binding of free fatty acid to human erythrocytes were studied by measuring the distribution of various amounts of [1-^{14}C]lauric acid between washed human erythrocytes and defatted human plasma albumin. Incubations were done at 37°C in an isotonic phosphate-buffered salt solution. Laurate uptake approached a steady state value within 1 hr of incubation over the range of laurate–albumin molar ratios that were tested. Uptake was due primarily to a transfer of laurate from albumin to the cell, not to incorporation of the intact laurate–albumin complex. The fatty acid binding sites of the erythrocyte are located predominantly on or within the cell membrane. The binding model which best fitted the laurate uptake data consisted of two classes of erythrocyte binding sites. This model contains a small number of sites, 2.0×10^{-13} moles/10^6 cells, that have an average apparent association constant of 1.8×10^6 M^{-1} for laurate. Thus, the average strength of these sites is of the same order of magnitude as the stronger laurate binding sites of albumin. The binding model also contains a relatively large number of weaker fatty acid binding sites, 1.3×10^{-11} moles/10^6 cells, that have an average apparent association constant of 1.3×10^4 M^{-1} for laurate. These sites are too weak to bind appreciable amounts of laurate unless the fatty acid–albumin molar ratio is elevated.

Supplementary key words albumin · binding sites · association constants · membranes

THE FIRST STEP in the utilization of free fatty acid by a mammalian cell is binding of the fatty acid in unesterified form to the cell membrane (1–3). Goodman (2) analyzed the binding reaction quantitatively, using washed human erythrocytes and palmitic acid. His analysis detected the presence of only a single class of erythrocyte binding sites, and these sites were located on the cell membrane. However, subsequent studies with human erythrocytes (4), Ehrlich cells (3), *Acanthamoeba* (5), and rat diaphragm (6) suggested that at least two pools of free fatty acid are associated with the cells. This conclusion was reached indirectly and is based upon the observation that only part of the free fatty acid which is taken up is exchangeable. In an attempt to clarify the apparent discrepancy between the direct binding analysis and the indirect exchangeability results, we have performed a study of the binding of lauric acid to human erythrocytes, using the procedures of Goodman (2). The reasons for selecting laurate rather than the commonly occurring longer-chain fatty acids are described below. Our investigations indicate that the human erythrocyte contains at least two classes of fatty acid binding sites, a result consistent with that predicted by the exchangeability data for these as well as other cells (3–6).

The procedure of Goodman for analysis of fatty acid binding to cells involves the assumption that when the steady state uptake is achieved, the unbound fatty acid anion concentration in the incubation medium is in equilibrium with both the fatty acid bound to albumin and that bound to the cell (2). This is represented by Equation 1:

$$\bar{v} \rightleftarrows c \rightleftarrows \bar{y} \qquad \text{(Eq. 1)}$$

where $\bar{v}$ is the molar ratio of fatty acid to albumin, c is the unbound fatty acid anion activity, and $\bar{y}$ is the amount of fatty acid bound to the cell. The initial step in the data analysis is to determine c as a function of $\bar{v}$. A serious com-

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plication is introduced into this procedure with fatty acids containing 14 or more carbon atoms because of the existence of fatty acid association (most probably anion dimerization) in aqueous solutions (7). Hence, the simplifying assumption made in albumin-binding studies (8, 9), as well as in the erythrocyte uptake study (2), that the total unbound fatty acid concentration is an accurate approximation of the unbound fatty acid anion activity is not valid for the longer-chain fatty acids. Since at least two forms of unbound anions (monomer and dimer) exist, and their ratio varies relative to fatty acid concentration (7), an accurate mathematical representation of the uptake data for fatty acids containing 14 or more carbon atoms becomes difficult to formulate. Although only small quantities of laurate are taken up by the erythrocyte relative to the longer fatty acids (10), we found that the uptake was of sufficient magnitude to permit accurate measurements. Moreover, in agreement with earlier reports (7, 11), our distribution analysis indicated that there was no appreciable association of laurate in aqueous solution over the range of laurate–albumin molar ratios that we employed. For these reasons, we selected laurate as an appropriate fatty acid ligand for our studies of the cell binding sites.

METHODS

Lauric acid was obtained from The Hormel Institute, Austin, Minn. [1-^{14}C]Lauric acid was purchased from New England Nuclear Corp., Boston, Mass. The labeled material was purified by extraction into alkaline ethanol, and the radiopurity of the resulting material was greater than 99% as determined by thin-layer chromatography (3). Crystalline human plasma albumin was obtained from Miles Laboratories, Kankakee, Ill., and it was extracted with activated charcoal (12) and dialyzed (9) in order to remove adsorbed impurities. Protein concentration was measured by the biuret method (13). ^{131}I-labeled human albumin was a gift from Dr. Frank Cheng of the Nuclear Medicine Section, Department of Radiology, University of Iowa. [1-^{14}C]Laurate–albumin solutions were prepared by addition of a warm solution of the sodium soap to the protein dissolved in a buffer solution containing 132 mM NaCl, 4.9 mM KCl, 1.2 mM $MgSO_4$, and 16 mM Na_2HPO_4 adjusted to pH 7.4 with 1 N HCl (3). This buffer was used throughout the study and is referred to in the text as "phosphate-buffered salt solution." Free fatty acid content was determined by titration (14); fatty acid radioactivity was measured in a Packard Tri-Carb 3375 liquid scintillation spectrometer, using a toluene–methanol scintillator solution (3). ^{131}I radioactivity was measured in a well scintillation counter.

The method employed for the measurement of the partition of [1-^{14}C]lauric acid between *n*-heptane and phosphate-buffered salt solution has been presented in complete detail (9). Experimental data for the binding of [1-^{14}C]lauric acid to human plasma albumin were obtained by procedures previously reported (9) and consisted of the total unbound fatty acid anion concentration, c, as a function of the molar ratio of bound fatty acid to albumin, $\bar{\nu}$. The data were initially analyzed in terms of a Scatchard model:

$$\bar{\nu} = \sum_{i=1}^{m} \frac{N_i k_i' c}{1 + k_i' c} \qquad \text{(Eq. 2)}$$

where m is the number of classes of binding sites, N_i is the number of binding sites in class i, and k_i' is the average apparent association constant for that class. Preliminary estimates of the equilibrium constants were then calculated from the results of this analysis. Using these calculated estimates as starting values, the experimental value of $\bar{\nu}$ for each value of c was refitted to the stepwise equilibrium model:

$$\bar{\nu} = \frac{K_1 c + 2K_1K_2c^2 + \ldots + nK_1K_2\ldots K_n c^n}{1 + K_1 c + K_1K_2c^2 + \ldots + K_1K_2\ldots K_n c^n} \qquad \text{(Eq. 3)}$$

where the unknown parameters K_1, K_2, ..., K_n represent the equilibrium constants for the binding of the ligand to the macromolecule. The preference for Equation 3 as opposed to Equation 2, as well as a description of the curve-fitting procedure employed, has been presented in detail (15, 16).

Erythrocytes were isolated from freshly drawn venous blood donated by normal, fasting human volunteers. EDTA was employed as the anticoagulant. The blood was transferred to a chilled polyethylene tube, and the cells were sedimented at 300 *g* for 10 min at 0°C. After the plasma was siphoned off, the upper 20% of the cell pellet was discarded and the remaining erythrocytes were resuspended in phosphate-buffered salt solution and resedimented. The washing procedure was repeated three times. The erythrocytes were then suspended in phosphate-buffered salt solution, and cell counts were made with a clinical hemocytometer and microscope. White blood cells were not detected in these erythrocyte suspensions. Approximately 2×10^9 erythrocytes were incubated in each flask with laurate–albumin solution. Incubation was at 37°C in a water bath incubator with shaking at 60 oscillations/min. Air served as the gas phase. After incubation, the cells were sedimented and washed three times with phosphate-buffered salt solution (3). Lipid was extracted from the erythrocytes with isopropanol and chloroform by the method of Rose and Oklander (17). The lipid extract was dissolved in chloroform and separated by thin-layer chromatography on Adsorbosil-5 in a solvent system containing hexane–diethyl ether–methanol–acetic acid 180:40:4:6 (3). The chromatogram was stained with I_2, and the segment of

the gel corresponding to the region in which the free fatty acid migrated was outlined. After the I_2 had sublimed, the radioactivity present in this segment of the gel was measured using a dioxane-containing scintillator solution (18). Quenching was monitored with the external standard.

The experimental data for the binding of laurate to human erythrocytes consisted of measurements of the molar ratio of fatty acid to albumin after the steady state was achieved, $\bar{v}$, and of the amount of fatty acid associated with the cells, $\bar{y}$. For each value of $\bar{v}$, the corresponding value for the unbound fatty acid concentration, c, was calculated by inverting Equation 3. This was done by substituting into Equation 3 the equilibrium constants, K_i, calculated from the laurate–albumin binding data, and using a Newton-Raphson iterative technique to determine c for each value of $\bar{v}$. This determination is unique because Equation 3 is a strictly increasing function of c, and the numerical results can be calculated to an accuracy within the experimental error of the data. These values of $\bar{y}$ and c were then fitted to the Goodman model (2):

$$\bar{y} = \sum_{i=1}^{m} \frac{R_i k_i c}{1 + k_i c} \qquad \text{(Eq. 4)}$$

where m is the number of classes of binding sites, R_i is the number of moles of binding sites in class i per 10^6 cells, and k_i is the average apparent association constant for that class. The fitting was done on an IBM System/370 computer using a least-squares model fitting procedure in conjunction with an IBM 2250 graphic display unit.

RESULTS

Lauric acid partition

Fig. 1 illustrates the distribution of [1-^{14}C]lauric acid between n-heptane and phosphate-buffered salt solution, pH 7.4, at 37°C. These data were well fitted to the partition equation described by Goodman (11), without the need for any aqueous phase association term (7). The value utilized for the partition ratio at infinite dilution (K_p) was 3, that for dimerization in the heptane phase (k_d) was 7×10^3, and that for pK_a was 4.8. These values are identical to those calculated by Goodman for lauric acid (11).

Lauric acid binding to albumin

Fig. 2 illustrates the binding of [1-^{14}C]lauric acid to defatted, crystalline human plasma albumin, dissolved in phosphate-buffered salt solution, pH 7.4, at 37°C. The molar ratio of laurate to albumin, $\bar{v}$, is plotted as a function of the negative logarithm of the unbound laurate concentration, c. Since the pK_a of lauric acid is 4.8 and the partition data indicated that there is no appreciable

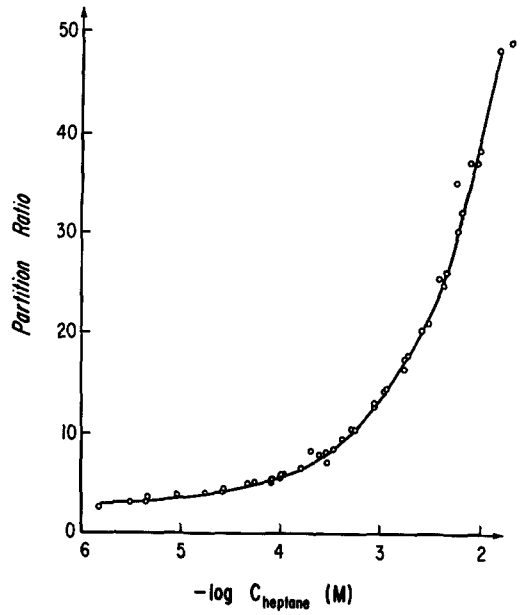

FIG. 1. Partition of [1-^{14}C]lauric acid between n-heptane and phosphate-buffered salt solution, pH 7.4. The negative logarithm of the concentration of laurate in the heptane phase is plotted on the abscissa; the ratio of laurate in the heptane vs. aqueous phase is plotted on the ordinate. Incubation was at 37°C. These data were obtained from four separate incubations.

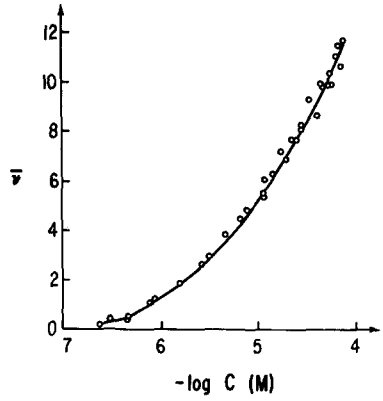

FIG. 2. Binding of [1-^{14}C]lauric acid to human serum albumin. Incubation was at 37°C in phosphate-buffered salt solution, pH 7.4. The negative logarithm of the unbound laurate concentration is plotted on the abscissa; the molar ratio of laurate to albumin is plotted on the ordinate. These data were obtained from two separate incubations.

anion association over this range of concentrations, the values of c were calculated by assuming that the total unbound laurate concentration is an accurate approximation of the laurate anion activity. These data initially were fitted to a Scatchard binding model (Equation 2) containing three classes of albumin binding sites (9), with $N_1 = 3$, $k_1' = 5.1 \times 10^5$ M^{-1}; $N_2 = 8$, $k_2' = 5.0 \times 10^4$ M^{-1}; and $N_3 = 27$, $k_3' = 1.3 \times 10^3$ M^{-1}. Preliminary estimates of the equilibrium constants were then calculated from the results of this analysis. Using these

calculated estimates as starting values, the data were refitted to the stepwise equilibrium model (Equation 3) (15, 16). The best fit to this model was obtained when the first six equilibrium constants were: $K_1 = 2.0 \times 10^6$ M^{-1}, $K_2 = 9.0 \times 10^5$ M^{-1}, $K_3 = 2.6 \times 10^5$ M^{-1}, $K_4 = 2.6 \times 10^5$ M^{-1}, $K_5 = 2.5 \times 10^5$ M^{-1}, and $K_6 = 4.2 \times 10^4$ M^{-1}.

Lauric acid uptake by erythrocytes

The time course of [1-^{14}C]lauric acid uptake in unesterified form by suspensions of washed erythrocytes is shown in Fig. 3. At each of the three laurate–albumin molar ratios that were tested, the uptake approached a steady state value within 1 hr of incubation. Therefore, this incubation time was selected for all of the subsequent experiments.

Fig. 4 illustrates the uptake of [1-^{14}C]laurate by intact erythrocytes relative to the molar ratio of laurate to albumin in the medium at the end of the incubation (i.e., after 1 hr of incubation when the steady state uptake was achieved). Laurate uptake increased markedly as the molar ratio was raised.

Additional studies with [1-^{14}C]lauric acid complexed with ^{131}I-labeled albumin were done in order to determine whether laurate uptake was due primarily to laurate transfer from albumin to the cells or from uptake of the intact laurate–albumin complex by the cells. The data obtained from analyses of the medium before and after incubation are listed in Table 1. From 32 to 37% of the [1-^{14}C]laurate was taken up by the cells under these conditions. In contrast, only very small amounts of the available albumin were taken up. In additional double-label experiments, the cells were isolated and analyzed directly for ^{14}C and ^{131}I uptake. We observed a similarly large uptake of ^{14}C-labeled laurate, but the ^{131}I content of the cells was not significantly different from background radioactivity. From these data we conclude that the laurate uptake was due almost exclusively to transfer from albumin to the erythrocyte, not from uptake of the intact laurate–albumin complex.

Membranes (ghosts) prepared from erythrocytes by hemolysis also took up laurate from these incubation media. The average uptake in four experiments in which the laurate–albumin molar ratio was 4 (2.1 ± 0.6 nmoles) was of the same order of magnitude as that obtained when intact erythrocytes were incubated with a solution of molar ratio 4 (see Fig. 4). The [1-^{14}C]laurate uptake of ghosts incubated in a medium in which the laurate–albumin molar ratio was 8 (4.4 ± 0.7 nmoles) also was of the same order of magnitude as that obtained with intact erythrocytes incubated in a medium in which the molar ratio was 8. Only trace quantities of laurate were bound to the membrane-free hemolysate when it was equili-

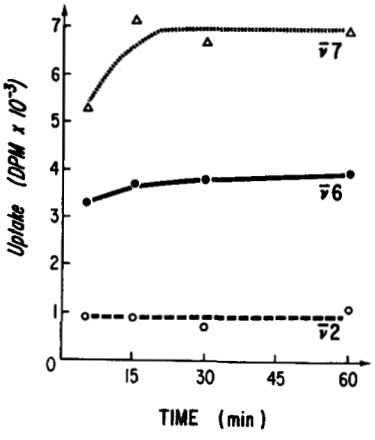

FIG. 3. Time course of [1-^{14}C]lauric acid uptake by human erythrocytes. Uptake refers only to the quantity of laurate radioactivity recovered from the erythrocytes as free fatty acid. Incubations were at 37°C in phosphate-buffered salt solution, pH 7.4, containing 0.2 mM human serum albumin and various amounts of laurate. The data at each molar ratio were obtained with a different preparation of cells. Each point is the mean of two determinations.

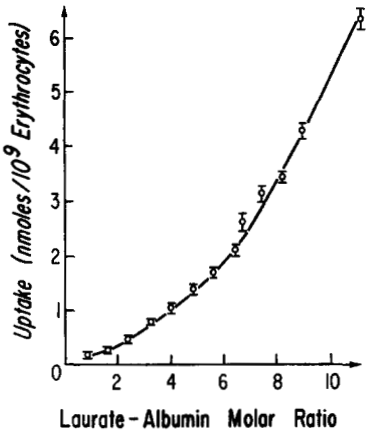

FIG. 4. Uptake of [1-^{14}C]lauric acid by human erythrocytes relative to the molar ratio of laurate to albumin in the incubation medium. Conditions of incubation are identical with those listed under Fig. 3. Seven different preparations of cells were employed to obtain these data. Each value is the mean ± SE of 6–10 determinations.

TABLE 1. Comparison of the uptake of [1-^{14}C]laurate and ^{131}I-labeled albumin by erythrocytes[a]

Parameter	Experiment Number			
	1	2	3	4
Medium laurate–albumin ratio				
Before incubation	0.033	0.034	0.034	0.037
After incubation	0.023	0.022	0.023	0.024
Laurate uptake (%)	32	37	33	35
Albumin uptake (%)	1	3	0[b]	0[b]

[a] Incubation was for 60 min at 37°C.
[b] The ^{131}I radioactivity in these samples did not differ significantly from background.

brated with [1-^{14}C]laurate dissolved in *n*-heptane. These data, in agreement with the observations of Goodman (2), indicate that the fatty acid binding sites of the erythrocyte probably are located on or within the cell membrane.

Analysis of laurate binding to erythrocytes

The values for the unbound laurate concentration, c, were calculated at each of the steady state laurate–albumin molar ratios shown in Fig. 4. This was done by substituting into Equation 3 the stepwise equilibrium constants, K_i, calculated from the lauric acid–human albumin binding data. Laurate uptake, $\bar{y}$, was then expressed as a function of c, and these data were graphed in the form of a Scatchard plot (19) as shown in Fig. 5. The values of $\bar{y}$ and c were then fitted to the Goodman model (Equation 4), using the computerized curve-fitting routines. The first step in the fitting procedure was to decide upon the number of classes of erythrocyte binding sites. The results of trial fittings of Equation 4 for $m = 1$, 2, and 3 are given in Table 2. In going from one to two classes of binding sites, the sum of squares was reduced considerably; this finding was consistent with the observation that the data in Fig. 5 cannot be accurately fitted to a straight line. Adding a third class of erythrocyte sites improved the sum of squares only slightly, but it introduced much greater uncertainties in the derived parameters as indicated by the large increase in the average condition number. These results indicate that the model containing two classes of erythrocyte binding sites provides the most appropriate least-squares fit of these data.

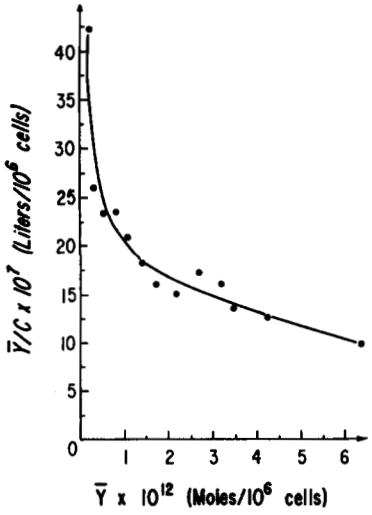

FIG. 5. Scatchard-type plot of the lauric acid uptake data shown in Fig. 4. The parameter c refers to the molar unbound laurate concentration that is in equilibrium with the laurate bound to the cells; $\bar{y}$ is the quantity of laurate bound to the cells (moles/10^6 cells).

TABLE 2. Models for binding of lauric acid to human erythrocytes

Parameter	Classes of Erythrocyte Binding Sites		
	1	2[a]	3
R_1[b] (moles/10^6 cells)	1.22×10^{-11}	2.01×10^{-13}	7.92×10^{-14}
k_1[c] (M^{-1})	1.65×10^4	1.76×10^6	4.38×10^{12}
R_2[b] (moles/10^6 cells)		1.33×10^{-11}	5.39×10^{-12}
k_2[c] (M^{-1})		1.32×10^4	3.04×10^4
R_3[b] (moles/10^6 cells)			5.99×10^{-11}
k_3[c] (M^{-1})			7.36×10^2
Sum of squares	0.210	0.153	0.151
Root-mean-square error	0.138	0.130	0.147
Average condition number	2.3×10	5.7×10	8.4×10^2

[a] Model selected as the best fit to the experimental data.
[b] Number of binding sites in this class.
[c] Average apparent association constant of the binding sites in this class.

DISCUSSION

Our results indicate that the human erythrocyte contains at least two classes of fatty acid binding sites. The sites are located on or within the cell membrane. The stronger class has approximately 2.01×10^{-13} moles of binding sites/10^6 cells, or 1.2×10^5 sites/cell. The average apparent association constant of laurate and these sites is 1.8×10^6 M^{-1}. Hence, this small class of strong sites has an association constant for laurate that is similar in magnitude to the stronger binding sites of human albumin. These strong sites probably bind much of the "nonexchangeable" or "irreversibly bound" free fatty acid pool of the cell (3–6). The large, weaker class of binding sites contains approximately 1.33×10^{-11} moles of sites/10^6 erythrocytes, or 8.0×10^6 sites/cell. These binding sites have an average apparent association constant for laurate of 1.3×10^4 M^{-1}. Exposure of the cell to fatty acid-poor albumin would be expected to remove much of the fatty acid associated with these weaker binding sites, and the fatty acid present at these sites probably represents the exchangeable or reversibly-bound free fatty acid pool of the cell (3–5).

The qualitative aspects of laurate binding to the erythrocyte are similar to those noted with palmitate (2, 4). However, laurate uptake over the range of fatty acid–albumin molar ratios examined was much smaller. At a given molar ratio, many more erythrocyte binding sites would be occupied if longer-chain fatty acids such as palmitate were utilized rather than laurate. We attempted to prepare laurate–albumin solutions of molar ratio greater than 12 in order to study a wider range of up-

takes, but this was unsuccessful because of solubility limitations. Therefore, it is possible that additional erythrocyte binding sites exist but were not detected because a sufficiently wide range of fatty acid uptakes could not be observed using laurate as the test substance.

We have independently analyzed, using our methods, the palmitate uptake data reported by Goodman and have derived the same one-class binding model that he described (2). Therefore, the difference between the model that we derived using the lauric acid data and the one developed by Goodman using palmitic acid does not involve the analytical procedures. The possibility that the erythrocyte may interact differently with laurate and palmitate certainly must be considered. For example, laurate, because of its smaller size, may be able to interact with certain sites that are unavailable to palmitate. If this were the case, then the binding model derived using the palmitate uptake data would be much more relevant from the physiological viewpoint because most of the plasma fatty acids contain 16 or more carbon atoms. However, before accepting this conclusion, the question of palmitate dimerization in aqueous media must be considered (7). Dimerization corrections were not made in the palmitate study because this phenomenon was then unknown. Our studies indicate that dimerization becomes appreciable only at high palmitate–albumin molar ratios, i.e., when the unbound palmitate concentration is high (16). Yet, it is precisely these data points, the uptake values ($\bar{y}$) that occur at high values of $\bar{v}$ and c, that are crucial in determining whether the cell binding curve is resolvable into one or more components. The existence of at least two classes of erythrocyte binding sites is consistent with the results that Shohet, Nathan, and Karnovsky obtained using palmitate (4). These investigators noted that although the bulk of the newly incorporated palmitate was exchangeable, a small amount of the unesterified palmitate associated with the erythrocyte could not be removed by a single exposure to albumin.

The binding model that we have derived from the laurate data produces a picture of the cellular free fatty acid uptake process that differs in one major respect from the currently accepted view. According to Goodman's model (2), the cell binding sites have an affinity for fatty acid that is at least one order of magnitude less than that of the strong albumin binding sites. Transfer of fatty acid from albumin to the cell is explained on the basis of rapid metabolism of the fatty acid that associates with the cell. In other words, the rapid removal of fatty acid from the cell binding sites provides a concentration gradient that is sufficient to cause fatty acid to flow in the direction of the cell, even when the free fatty acid–albumin molar ratio is low. In contrast, our two-class model indicates that the erythrocyte contains a relatively small number of membrane sites that bind fatty acid tightly. These sites would be expected to enhance fatty acid movement from albumin to the cell. Since our uptake measurements were made after equilibrium was achieved, they provide no information concerning the mechanism through which fatty acid is taken up by the binding sites. Each class of sites may compete independently and simultaneously for fatty acid, or fatty acid may bind initially to one class of sites and then be transferred to the other. The latter interpretation is compatible with the work of Shohet et al. (4) that indicated that palmitate passes from weak sites at the cell surface to stronger internal binding sites. Because of the many similarities between our data and those obtained with palmitate, we believe that this two-class binding model probably is applicable to the physiologically important long-chain fatty acids. However, the possibility remains that it applies only to laurate or similar medium-chain fatty acids.

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