Palmitate Uptake by Hepatocyte Monolayers

Effect of Albumin Binding

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Abstract

The uptake of ^{14}C-palmitate by rat liver cell monolayers is depressed by binding of the fatty acid to albumin. When the uptake flux is divided by the concentration of free palmitate in the bathing medium, however, the resulting clearance is ~14 times greater in the presence of albumin than in its absence. These findings are not accounted for by the different diffusion rates of free and bound palmitate across an unstirred fluid layer, nor attributable to nonequilibrium binding. Instead we argue that the most plausible explanation is accelerated dissociation of albumin-palmitate complexes mediated by the cell surface—an interpretation that also explains the uptake kinetics of other albumin-bound organic anions by perfused rat liver.

Introduction

The role of albumin binding in the hepatic uptake of organic anions has become controversial recently after many years of relatively little interest. The controversy arises from recent reports (1–6) that the steady state removal of taurocholate, rose bengal, and oleic acid by perfused rat liver is much faster than predicted by the traditional teaching that only the free or unbound form of these solutes is available for hepatic uptake. The inferences obtained with perfused livers, however, depend on simplifications required to deal with the unknown distributions of transport capacity and surface to volume ratio along the sinusoids, the need to infer binding reaction rates in the vascular lumen from measurements conducted in vitro, and the assumption that efflux of labeled solute from cytoplasm to extracellular fluid is independent of perfusate albumin concentration.

The present experiments were designed to avoid these uncertainties by measuring the uptake of palmitate by hepatocyte monolayers in the presence and absence of bovine[1] albumin. The experimental design includes vigorous stirring to minimize diffusive artifacts and a sufficiently large volume of bathing medium to ensure that palmitate uptake does not disturb the binding equilibrium in extracellular fluid. Only initial uptake rates are analyzed, moreover, to exclude a contribution by efflux.

The results show that the clearance of bound palmitate is much faster than it would be if only free fatty acid were available for uptake. A diffusive artifact attributable to unstirred layers adjacent to the cell surface is considered and largely excluded.

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1. The effect of rat albumin and of other proteins has been considered elsewhere (4, 5), though not definitely resolved. This specificity issue is beyond the scope of the present report.

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Methods

Hepatocyte monolayers. The livers of donor rats (Sprague-Dawley females, liver weight ~7 g) were perfused in situ at 15 ml min^{-1}, first with oxygenated Swims S-77 medium containing 0.5 mM EDTA and subsequently with oxygenated Swims S-77 medium containing 50 mg dl^{-1} collagenase and 5 mM $CaCl_2$. Livers were then removed, combed free of connective tissue, and filtered through nylon mesh. The resulting cell suspension was washed four times in perfusion medium then resuspended (5×10^5 cells ml^{-1}) in Ham's F-12 medium containing 10% fetal calf serum, streptomycin (100 μg ml^{-1}), penicillin G (100 U ml^{-1}), and insulin (0.1 U ml^{-1}). Portions of this suspension (1.5 ml, 7.5×10^5 cells) were placed in 35-mm plastic culture dishes previously coated with rat tail collagen and incubated at 37°C and 40 mm Hg P_{CO_2}. After 4 h the plates were washed free of nonadhering cells and the medium replaced with fresh Ham's F-12 including the additions listed above except that fetal calf serum was reduced to 5%. Subsequent changes of the same medium were at 24-h intervals but the fetal calf serum was omitted. As an index of cell membrane integrity we measured LDH activity of the cells and medium. The medium content of this enzyme was on the average 5% of the cell content on the same day that we measured palmitate uptake.

Palmitate uptake. 2-d cultures prepared from a single rat were used for each experiment. Monolayers were washed free of incubation medium in calcium-free Kreb's-Ringer phosphate buffer, drained, then immersed for 10–50 s in 250 ml of stirred Kreb's-Ringer phosphate buffer (pH 7.4, 37°C, 200 rpm) containing 0.2 μM ^{14}C-palmitate. Uptake was stopped by removing the monolayers from this "uptake" solution followed immediately by immersion for 4 s in 400 ml of stirred Kreb's-Ringer phosphate buffer (0°C, "stop" solution). Three monolayers were exposed to ^{14}C-palmitate for each of five time intervals (10, 20, 30, 40, and 50 s). One set of 15 monolayers was exposed in this way to albumin free uptake solution. Another set of 15 was immersed in an otherwise identical solution containing 25 μM bovine albumin. Each experiment thus yielded three replicate estimates of palmitate uptake at each of five time intervals in the presence and absence of albumin. To avoid bias attributable to the order of individual uptake determinations, we varied the sequence of the time intervals as well as the sequence in which we used albumin containing or albumin-free media. We used hepatocytes from six rats to conduct six experiments—a total of 180 uptake determinations in all. To ensure efficient stirring, plates were immersed in the uptake and stop solutions in such a way that the convective stream was directed toward the monolayer surface. This procedure did not dislodge cells from the monolayers which covered ~95% of the plate surface.

After immersion in stop solution, plates were inverted on absorbent paper to drain as much extracellular fluid as possible. To each experimental plate as well as to a control plate that contained 500 μg of albumin but no cells, we added 0.6 ml of 2 N NaOH to digest the cells. After incubation of this mixture at 60°C for 20 min and subsequent neutralization with HCl, we measured the radioactivity and total protein by liquid scintillation counting and the Lowry (7) procedure, respectively. The control plate served as background for the radioactivity measurement and as a standard in the Lowry procedure. Total and free radioactivities of the uptake solution were measured at the beginning and at frequent intervals during each experiment. Blank plates coated with collagen but containing no cells were carried through the same procedure to measure the time course of palmitate binding to the collagen coat.

Palmitate binding. The distribution of radioactivity between the uptake solution and *n*-heptane served to measure the free fraction of pal-

mitate. To this end, 1-ml portions of medium and heptane were placed in a glass tube together with a glass bead that facilitated stirring. The tubes were gassed with N_2, sealed, and placed in a shaking water bath (37°C) for 48 h, preliminary experiments having verified that the partition ratio was stable after 24 h. The equilibrium distribution was determined from samples of the organic and aqueous phases, the latter removed without heptane contamination by a modification of the method described by Spector (8). A small polyethylene tube was inserted through the heptane layer into the aqueous layer using a stream of air to keep heptane from entering the tube. A sample of the aqueous layer was subsequently obtained by siphon after discarding the initial effluent to remove any residual heptane. The free fraction, α, of palmitate in albumin-containing medium was computed as $\alpha = PR_+/PR_-$, where PR denotes the heptane/aqueous partition ratio and the subscripts, + and −, denote the presence and absence of albumin, respectively.

Denaturation of albumin by heptane was excluded by measuring the binding of rose bengal to albumin before and after subjecting the protein to the heptane partition procedure. We used a spectrophotometric method described previously (2) to measure the binding of rose bengal and to verify that this dye competes with palmitate for binding sites on albumin. Shaking albumin with heptane for 48 h did not alter the binding of rose bengal.

Materials. The polystyrene tissue culture plates were purchased from Vangard International, Inc., Neptune, NJ. The collagenase came from Cooper Biomedical, Inc., Malvern, PA. Rat tail collagen was a gift from D. R. LaBrecque, University of Iowa, Iowa City, IA. The other tissue culture supplies came from Gibco, Grand Island, NY. Lactic acid dehydrogenase activities were measured with an assay kit from Sigma Chemical Co., St. Louis, MO. Armour Pharmaceuticals, Kankakee, IL, supplied the fatty acid free bovine albumin. Labeled palmitate (radiopurity > 99%) was purchased from ICN, Irvine, CA, and rose bengal from Accurate Chemical & Scientific Corp., Hicksville, NY.

Calculations. Curve fitting was carried out on an IBM PC/XT computer with an iterative, nonlinear, derivative-free least squares algorithm (9). Goodness of fit is reported as the coefficient of determination.

Results

Fig. 1 illustrates a typical uptake experiment. A summary of the transport and binding data from all six such experiments appears in Table I. Notable features are the extensive binding of palmitate to albumin and the marked depression of palmitate clearance with which this binding is associated. It is apparent, however, that albumin produces a much smaller reduction in palmitate clearance than it does in the concentration of free fatty acid. Accordingly, when palmitate uptake is referred to the concentration of free palmitate instead of to the total concentration, the apparent clearance of free palmitate (i.e., total clearance divided by free fraction) is much higher in the presence of albumin than in its absence. This is the meaning of the striking difference in the slopes displayed in Fig. 1 and of the nearly 14-fold difference in the apparent clearances displayed in the last column of Table I.

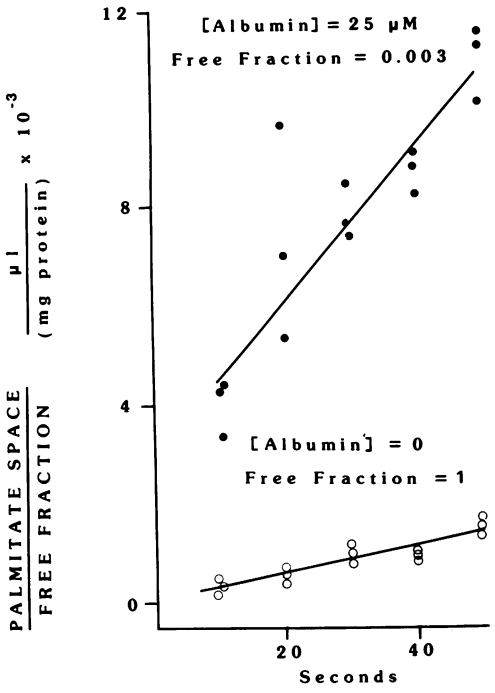

Figure 1. Palmitate uptake in the presence (●) and absence (○) of albumin. The slopes depict the apparent clearances of free palmitate.

The results from three blank experiments carried out in the absence of liver cells appear in Fig. 2. Adsorption of both bound and free palmitate to the collagen coat is complete within 10 s, i.e., the uptake slope beyond this point is indistinguishable from zero. However, the ordinate intercept of this asymptote is much higher when the palmitate is free than when it is bound to albumin. Thus, free palmitate binds to collagen much more avidly than do albumin-palmitate complexes. This result also shows that most of the palmitate recovered from these plates is bound to the collagen layer, and not simply present in retained extracellular fluid. The ordinate values in Fig. 2 are in microliters rather than in microliters per milligram because the protein in the collagen layer is too small to measure by the Lowry procedure.

To be sure that the clearance of free palmitate by liver cells does not depend on fatty acid concentration, we conducted three additional experiments to measure the removal of palmitate from albumin-free buffer. These clearances are 29.4 and 31.4 $\mu l\ s^{-1}$

Table I.

Albumin	Palmitate	Partition ratio	Free fraction	Palmitate clearance	Apparent* free clearance
μM	μM			$\mu l\ s^{-1}\ mg^{-1}$	$\mu l\ s^{-1}\ mg^{-1}$
0	0.2	150±8	1	25±4	25±4
25	0.2	0.3±0.18	0.002±0.001	0.6±0.1	348±229

Milligram refers to milligram of cell protein (average value = 0.57±0.09 mg/plate). ± denotes the 95% confidence interval. To compute the predicted clearance of free palmitate in the presence of albumin, multiply the clearance determined in the absence of albumin (=25 $\mu l\ s^{-1}\ mg^{-1}$) by the free fraction (=0.002) to obtain 0.05 $\mu l\ s^{-1}\ mg^{-1}$. This value may then be compared with the observed clearance (=0.6 $\mu l\ s^{-1}\ mg^{-1}$). * Apparent free clearance equals clearance divided by free fraction.

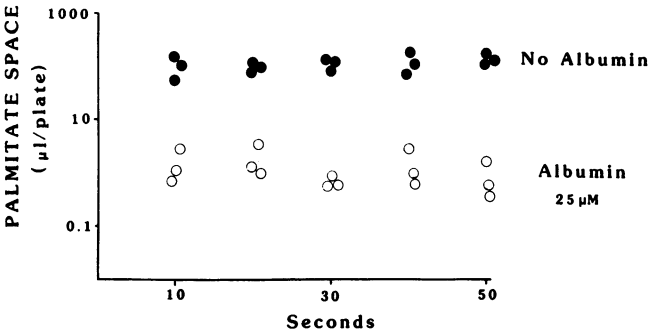

Figure 2. The time course of palmitate adsorption to the collagen layer in cell-free plates. Regression equations±95% confidence intervals: ○, $y = (-0.014 \pm 0.033)x + (1.66 \pm 1.09)$; ●, $y = (1.02 \pm 1.30)x + (82 \pm 43)$.

mg^{-1} at palmitate concentrations of 0.2 and 0.02 μM, respectively. It follows that in the range of interest here the clearance of free palmitate is independent of its concentration. This detail is important because if palmitate uptake were to display saturation kinetics, a larger value of apparent free clearance might be found in the presence of albumin than in its absence, owing to the much smaller concentration of free fatty acid in the presence of albumin.

Because the molar ratio of palmitate to albumin in our experiments is only 0.008 (=0.2/25), the equilibrium free fraction, α, derived from the binding reaction at a single high affinity site, is given by

$$\alpha = 1/(K_L P + 1), \tag{1}$$

where K_L is the equilibrium constant and P is the concentration of albumin. From the values of α and P in Table I, Eq. 1 predicts $K_L = 2 \times 10^7\ M^{-1}$, which is similar to the value for the highest affinity site identified by Spector ($3 \times 10^7\ M^{-1}$) (8).

In our interpretation of these results, we use the equilibrium free fraction to calculate the concentration of unbound palmitate in albumin-containing medium. For this reason it is important to establish that the binding of palmitate to albumin was effectively at equilibrium even though fatty acid was being removed from the medium by liver cells. To appreciate the basis for this conclusion, consider the following calculation:[2]

$$\text{fraction of free palmitate removed} = \frac{\text{(mass of free palmitate removed during 50 s)}}{\text{(volume of extracellular fluid)} \times \text{(palmitate concentration)(free fraction)}}$$

$$= \frac{\text{[(uptake slope)(50 s) + uptake intercept](cell protein)}}{\text{(volume of extracellular fluid)(free fraction)}}$$

$$= \frac{[(0.58\ \mu l\ s^{-1}\ mg^{-1})(50\ s) + (5.91\ \mu l\ mg^{-1})](0.57\ mg)}{(250 \times 10^3\ \mu l)(2.3 \times 10^{-3})}$$

$$= 0.03.$$

Thus, during even the longest clearance interval, hepatocytes removed only 3% of the available free palmitate. It follows that only a trivial perturbation of the binding equilibrium occurred, and that the free fraction determined from equilibrium partition ratios is a conservative basis from which to calculate the apparent clearance of free palmitate.

Discussion

The simplest interpretation of our results can be appreciated by writing the conventional relation between uptake flux and substrate concentration on the assumption that only free palmitate is available for transport. This relation is:

$$J/\alpha u = K, \tag{2}$$

where J is the uptake rate, α is the free fraction, u is the palmitate concentration, and K is the intrinsic clearance of free fatty acid. If K is a constant independent of the concentrations of albumin and of free palmitate, Eq. 2 predicts that the slopes in Fig. 1 should be equal. But our finding is that K in the presence of albumin ($\alpha = 0.002$) is ~14 times higher than in its absence ($\alpha = 1$). Accordingly, the assumption that leads to Eq. 2 must be wrong. Specifically, our results suggest that bound as well as free palmitate is acting as substrate for uptake. Because albumin is not itself removed,[3] we argue that sites on the liver cell surface catalyze the dissociation of albumin-palmitate complexes in such a way as to ensure that the liberated free fatty acid is available for uptake before it reenters the pool of free palmitate in extracellular fluid. This is the interpretation we have suggested previously from the uptake kinetics of other bound anions in perfused livers (6). An alternate interpretation invoking a diffusive artifact attributable to unstirred layers is theoretically tenable, however, and we therefore proceed to consider this possibility. Our approach to this objective is to derive the transport equation that includes an unstirred layer. We then apply the results obtained from the uptake of palmitate by blank plates to determine a lower bound on the diffusive conductance imposed by this layer. Finally, the equation that includes the diffusive and membrane transport conductances in series is fitted to the monolayer uptake data to show that the transport conductance is an order of magnitude larger in the presence of albumin than in its absence. To develop this argument we assume that only free palmitate is available for transport, and that binding reactions between palmitate (or its albumin complex) and the cell surface are much faster than diffusion to this surface. The latter stipulation ensures that we may represent the amount of fatty acid bound to the cell surface as a volume of distribution to be added to the volume of unstirred extracellular fluid. To this end, let q and r be the volumes of distribution on the cell surface of free palmitate and albumin-palmitate complexes, respectively. As illustrated by the model in Fig. 3, we use a and b to represent the concentrations of free and bound palmitate in stirred extracellular fluid, and A and B to denote these concentrations in the unstirred layer of fluid adjacent to the cell surface. These definitions lead to the following conservation equations for free and bound palmitate in the unstirred layer:

2. The palmitate space at 50 s is calculated using the average linear regression coefficients from six plots of space vs. time. The average slope is 0.58 $\mu l\ s^{-1}\ mg^{-1}$, and the average intercept at $t = 0$ is 5.91 $\mu l\ mg^{-1}$.

3. This possibility is not addressed directly by the present experiments but appears remote in view of previous reports (1, 10) that the steady state hepatic extraction of labeled albumin is trivial. In the present experiments, moreover, <1% of the albumin is bound to palmitate.

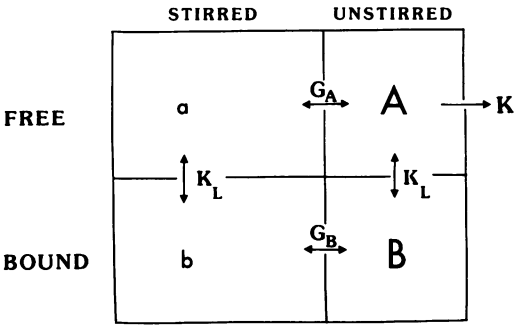

Figure 3. Compartmental model for predicting the effect of an unstirred layer of extracellular fluid. $G_A = D_A\phi/\delta$, $G_B = D_B\phi/\delta$.

$$(\phi\delta + q)(dA/dt) = (D_A\phi/\delta)(a - A) - KA \tag{3}$$

$$(\phi\delta + r)(dB/dt) = (D_B\phi/\delta)(b - B) \tag{4}$$

$$A(0) = B(0) = 0, \tag{5}$$

where δ and ϕ are respectively the thickness of the unstirred layer and its area, D_A and D_B are the aqueous diffusion coefficients of free and bound palmitate, and K is the membrane transfer coefficient (in units of volume per time) of free fatty acid. Recalling that the binding of palmitate to albumin is at equilibrium, we add Eqs. 3 and 4 to obtain

$$[\phi\delta + \alpha q + (1 - \alpha)r](dU/dt) + U(\theta + K\alpha) = u\theta \tag{6}$$

$$\theta = [\alpha D_A + (1 - \alpha)D_B]\phi/\delta, \tag{7}$$

where u $(= a + b)$ is the palmitate concentration in the stirred compartment, and U $(= A + B)$ is its concentration in the unstirred fluid layer. Integrating Eq. 6 with the boundary conditions in Eq. 5 leads to

$$U = u\theta/(\theta + \alpha K)[1 - exp\{-(\theta + \alpha K)t/v\}] \tag{8}$$

$$v = \phi\delta + \alpha q + (1 - \alpha)r. \tag{9}$$

To cast Eq. 8 in terms of the apparent space of free palmitate, let M be the amount of palmitate recovered from the monolayer at time t and let ρ be the fraction of v that is included with this recovery. M is thus the sum of two terms: the mass of intracellular palmitate, which we assume is recovered completely, plus an extracellular contaminant that is only partially removed by immersion in the stop solution. Specifically,

$$M = \alpha K \int_o^t U(\lambda)d\lambda + \rho vU. \tag{10}$$

Substituting for U, as defined by Eq. 8, and carrying out the indicated integration, we have

$$M/(u\alpha) = \{D_A + [(1 - \alpha)/\alpha]D_B\}\phi Kt/$$
$$(\{D_A + [(1 - \alpha)/\alpha]D_B\}\phi + \delta K) + v((\rho/\alpha)$$
$$- \theta K/(\theta + \alpha K)^2)(1 - exp\{-(\theta + \alpha K)t/v\}), \tag{11}$$

which is the palmitate space divided by the free fraction; i.e., the ordinate variable in Fig. 1.

Eq. 11 describes a monotonically rising curve with a slant asymptote whose slope is the coefficient of t in the first term. If $\delta = 0$, the slope of this asymptote is the intrinsic clearance, K, for all values of α. But for any particular value of $\delta > 0$, the slope increases as α declines. Moreover, the larger is δ the more important α becomes as a determinant of the slope. This behavior explains why an unstirred layer could be responsible for the difference in uptake slopes displayed in Fig. 1. To decide whether this possibility is the correct explanation, we seek to fit Eq. 11 to the uptake data obtained in the presence and absence of albumin. In particular, we ask whether the value of K recovered from this procedure when $\alpha = 1$ is similar to the fitted value of K when $\alpha = 0.002$. If K proves independent of α, the unstirred layer evidently explains the observations. If not, we conclude that the apparent difference in K is real, and accordingly that bound palmitate, as well as free, must serve as substrate for uptake. Eq. 11 contains too many unknowns (K, v, δ, ρ) to make this approach feasible directly. Estimates of δ are available, however, from the data obtained with blank plates, and ρ is constrained to lie between 0 and 1. The parameter vector in Eq. 11 is thus reducible to the two unknowns, K and v, which can then be determined by nonlinear regression.

Eq. 11 applied to blank plates simplifies to:

$$M/u = \rho'v'(1 - exp\{-\theta t/v'\}), \tag{12}$$

because the absence of cells ensures that $K = 0$. The prime is introduced in v' and ρ' to indicate that these variables may have different values than v and ρ defined earlier for the case where cells are present. The value of δ should be virtually the same in both situations, however, because monolayer plates and blank plates were subjected to identical stirring and because the microvilli on cells should make only a small contribution (~ 1 μm) to the total depth of the unstirred layer.

To make use of the observation (Fig. 2) that the uptake of palmitate by blank plates is complete by 10 s, consider first the experiments with albumin. Taking the ordinate intercept as a measure of $\rho'v'$ we have

$$1.66\ \mu l = \rho'v' = \rho'[\phi\delta + \alpha q' + (1 - \alpha)r'] > \rho'\phi\delta, \tag{13}$$

or

$$\delta < 1.66/\rho'\phi. \tag{14}$$

To set another upper bound on the unstirred layer thickness, we note that the absolute value of the exponent in Eq. 12 must be large to account for the observation that M/u is not a function of t for $t > 10$ s. Conservatively, we may set $1 - \exp - (\theta t/v') > 0.95$ at $t = 10$ s and obtain from Eq. 12

$$10\theta/v' > -\ln(0.05). \tag{15}$$

Considering the case where albumin is absent, we also have by analogy with Eq. 13

$$82\ \mu l = \rho'v'. \tag{16}$$

Eliminating v' between relations 15 and 16 and making use of Eq. 7 we obtain

$$\delta < [\alpha D_A + (1 - \alpha)D_B]10\phi\rho'/(-82 \ln(0.05)). \tag{17}$$

The inequalities 14 and 17 are of the form

$$\delta < C_1/\rho' \tag{18}$$

$$\delta < C_2\rho', \tag{19}$$

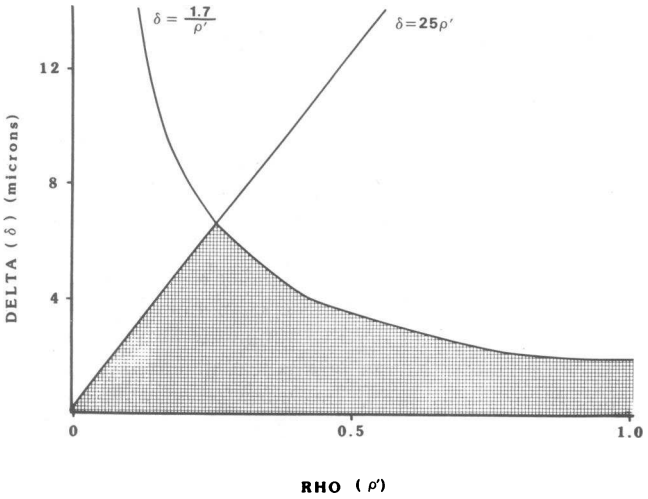

Figure 4. The shaded area contains all possible (δ, ρ') pairs defined by the inequalities $\delta < 1.7/\rho'$ and $\delta < 25\ \rho'$.

where C_1 and C_2 are constants equal to 1.7 and 25 μm, respectively.[4] The simultaneous solution of these inequalities is shown graphically in Fig. 4, where the shaded area denotes all possible values of δ for a given value of ρ'. The largest value of δ (=6.5 μm) corresponds to $\rho' = 0.26$. To eliminate the uncertainties in these estimates that arise from the scatter in Fig. 2, we compute an even more conservative upper bound on δ by using the 95% confidence intervals about the intercepts in Fig. 2. With this refinement, the upper bound on δ is 12 μm and the corresponding value of ρ' is 0.24. This value of δ is somewhat larger than the width of the Disse space in intact livers (~2 μm), but small compared with estimates of the unstirred layer reported for other epithelia studied in vivo (11). We attribute this difference to the anatomic simplifications that characterize monolayer cultures, and even more importantly to the convective effect of the fluid stream directed toward the monolayer surface.

Using the worst case value of δ and various arbitrary values of ρ between 0 and 1, we fitted Eq. 11 to each pair of monolayer uptake plots to compute the quotient of K when albumin was present to the value of K in its absence. The results, which appear in Table II, are independent[5] of ρ. The mean ratio of the K's is 9±2, and the mean coefficient of determination is 0.91. The true value of the clearance ratio may be substantially >9 inasmuch as we have used a very conservative estimate of δ.

There are two possible explanations for this result. One to be discussed further below is that the driving force for palmitate uptake includes bound as well as free fatty acid. The other is that the presence of albumin in the bathing medium greatly enhances the intrinsic permeability of the cell membrane to free palmitate. In considering this latter possibility, it should be recalled that all the monolayers were bathed by protein-free media

Table II. Apparent Intrinsic Clearance of Free Palmitate Corrected for the Unstirred Layer*

Albumin = 0	Albumin = 25 μM	Clearance ratio
19.1 (0.75)	188 (0.95)	9.84
21.7 (0.89)	156 (0.90)	7.19
18.1 (0.99)	138 (0.91)	7.62
15.8 (0.95)	186 (0.95)	11.77
16.9 (0.97)	121 (0.80)	7.16
17.5 (0.90)	185 (0.92)	10.57
		mean±SD = 9±2

Numbers in parentheses denote the coefficient of determination for each regression at $\rho = 0.25$ (see footnote 6).
* μl s^{-1} mg^{-1}.

for 24 h immediately before the measurements of palmitate uptake, and that as judged by leakiness to lactic acid dehydrogenase in this interval, their membrane integrity was unimpaired. Because the difference in apparent clearance of free palmitate is already apparent by 10 s, an albumin-induced increase in membrane permeability must occur rapidly, and it must respond to low concentrations of albumin (i.e., 0.025 mosM). We are unaware of a similar phenomenon reported elsewhere, nor can we suggest a mechanism that would account for it. Accordingly, we regard this possibility as remote although we cannot exclude it.

There are three other potential sources of misinterpretation that warrant additional comment. First, we used calcium-free media to minimize the confusion that might otherwise arise from the formation of Ca-palmitate soap. The absence of calcium, however, may have changed the permeability of the cell membrane. We cannot exclude this possibility or other changes in hepatocyte function that may occur when cells are removed from their normal environment. We are confident our results are relevant to normal liver function, nevertheless, because the effect of albumin we report is similar to that observed in whole livers not only with fatty acid (4) but with other anions as well (1–3, 5, 6).

Second, free long chain fatty acids may aggregate to form multimers above a critical aqueous concentration. If this occurred in our bathing medium the proportion of free palmitate present as monomers might be much greater in the presence of albumin than in its absence, because albumin reduces the free concentration. There are two reasons for discounting the possibility that the effect of albumin on palmitate uptake is related to such changes in the physical state of the free fatty acid. First, the aqueous solubility of palmitate monomers at pH 7.4 probably lies between 1 and 10 μM (12–14)—values that are 5–50 times higher than the concentration we have used. The second reason is that the clearance of free palmitate is invariant in the concentration range 0.02–0.2 μM. Thus, in our experiments, palmitate was entirely monomeric, or if not, multimers and monomers had equivalent molar clearance rates. The former interpretation is clearly the more reasonable one, but either way the effect of albumin binding is not attributable to the self-association of palmitate.

Finally, we consider whether the clearances we attribute to uptake might be distorted by loss of cell-associated palmitate

4. The free diffusion coefficients are $D_A = 6.5 \times 10^{-6}$ cm^2 s^{-1}, $D_B = 0.6 \times 10^{-6}$ cm^2 s^{-1}. ϕ computed for a circular plate 35 mm in diameter is 9.62 cm^2.

5. The effect of variations in ρ on the fitted values of K is not shown. The absence of any discernible effect may be predicted directly from Eq. 11, however, by noting that although ρ is a determinant of the asymptote intercept, it is not a parameter in the asymptotic slope.

during the wash procedure. Loss of radioactivity from the unstirred layer or from the cell surface is accounted for explicitly by incorporating ρ as an unknown parameter in the expression for M in Eq. 10, but efflux from within the cell has not been considered. We claim that even if such losses occurred, they have no effect on the final conclusion. To understand the logic of this position, recall that the wash procedure was identical for each monolayer and that palmitate was present at only tracer concentrations. If it occurred at all, therefore, efflux during the wash procedure led to the loss of a constant fraction of intracellular radioactivity. Under these circumstances, K in Eq. 3 and in those that follow can be considered as the uptake clearance reduced by a constant correction factor. Because our interpretation ultimately depends on the ratio of the uptake clearances, not on their individual values, the hypothetical efflux correction factor cancels out of their quotient. It follows that the magnitude of the efflux correction factor is immaterial to our conclusion. Note as an addendum to this argument that although efflux cannot be excluded, its contribution to the clearance slopes in Fig. 1 is probably negligible. There are two reasons. The wash interval was much shorter than even the shortest uptake interval, and the wash fluid was cold (0°C) whereas the uptake medium was warm (37°C).

In view of these considerations, we argue that the present report provides strong new evidence for mediated dissociation at the cell surface. To recapitulate an explanation presented elsewhere in detail (6), we envision sites on the plasma membrane that bind albumin-ligand complexes and catalyze their dissociation, presumably by causing a transient change in the conformation of albumin. Free ligand liberated in this process is subject to uptake directly from the cell surface without reentering the pool of free ligand in extracellular fluid. As a result, more free ligand is available for transport than one would infer from the concentration of free ligand in extracellular fluid.

It may appear at first glance that this interpretation is inconsistent with the finding that albumin produces a striking depression in total palmitate clearance—a finding that also calls into question whether mediated dissociation could be of any functional importance in promoting palmitate removal. There is an important feature of the monolayer experiments, however, that greatly amplifies the inhibiting effect of albumin beyond what one might expect to observe in an intact liver. The reason for this inhibiting effect and for its exaggeration in the present experiments is best appreciated from the model equation we have developed in a previous report (6). To predict the ratio of total clearances in the presence and absence of albumin, we assume that dissociation mediating sites on the cell surface bind free albumin and albumin-ligand complexes with the same affinity; i.e., we assume these sites respond to albumin, not to the ligand that it carries. Direct evidence for this view has already appeared in the case of rose bengal (15). We also assume that the uptake of free ligand from such sites is governed by the same rate constant that governs the uptake of free ligand directly from extracellular fluid. If the molar ratio of ligand to albumin is small, and if ligand binding is effectively at equilibrium, criteria that are both met here, the ratio of total clearances predicted by the model is

$$C_+/C_- = \alpha[1 + PK_LS_t/(P + K_s)]. \quad (20)$$

In this expression, C_+ and C_- denote total ligand clearances in the presence and absence of albumin, respectively, S_t is the concentration of cell surface sites (moles per volume of extracellular fluid), and K_s is the equilibrium constant for the binding of albumin to the cell surface. P, K_L, and α have the meanings defined by Eq. 1. In the special case where $S_t = 0$, Eq. 20 predicts, as expected, that $C_+/C_- = \alpha$. For another special case ($P = 0$, $\alpha = 1$), the clearance ratio is 1, also as expected. In all other situations, however, the clearance ratio exceeds α. Moreover, for particular values of P, K_L, and K_s, this difference depends critically on S_t. But S_t appears in Eq. 20 as a concentration, and therefore depends on the number of liver cells per unit of extracellular volume. Because the ratio of cells to extracellular fluid volume is on the order of a million times higher in a whole liver than it is in our monolayer preparations, the present experiments greatly exaggerate the competitive inhibitory effect of free albumin.

Finally it should be recalled that although albumin retards palmitate uptake, fatty acid removal proceeds at least nine times more rapidly than it would if there were not a mechanism at the cell surface to catalyze the dissociation of albumin-palmitate complexes. The survival value of this phenomenon is substantial, therefore, even though its effect is reduced by the special circumstances of the present experiments.

Summary

Rat liver cells in primary monolayer culture remove albumin-bound palmitate much faster than predicted by the conventional wisdom that only free palmitate is available for uptake. Diffusion artifacts account for less than a third of this discrepancy, which instead appears attributable to cell surface-mediated dissociation of albumin-palmitate complexes. The quantitative effect of this phenomenon is much less striking in monolayer preparations than it would be in intact livers, owing to the small number of liver cells that are present relative to the volume of extracellular fluid. The present results are similar to those reported earlier for taurocholate and rose bengal obtained with perfused rat livers. Because the kinetics of palmitate uptake are measured in the presence of equilibrium binding and in the absence of blood flow, however, the interpretation entails many fewer assumptions.

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