Mechanism for binding of fatty acids to hepatocyte plasma membranes

Robert B. Cooper, Noa Noy, and David Zakim

Division of Digestive Diseases, Department of Medicine, Cornell University Medical College, New York, NY 10021

Abstract The purpose of this study was to examine the interaction between fatty acids and plasma membranes from liver cells. We were unable to reproduce the reported effect of heating on the capacity of these membranes to bind [^{3}H]oleate (Stremmel et al. 1985 *Proc. Natl. Acad. Sci. USA.* **82:** 4–8). In fact, the distribution of [^{3}H]oleate between plasma membranes and unilamellar vesicles of lipids extracted from these membranes was in favor of the lipids, indicating the absence of a detectable amount of binding to a putative fatty acid binding protein in plasma membranes. Radius of curvature of vesicles (125 Å vs 475 Å) had no effect on the partitioning of fatty acid. In addition, the distribution of [^{3}H]oleate between plasma membranes and other phases had the properties of a partition coefficient over a 200-fold range of [^{3}H]oleate. There was no evidence in this experiment for a binding isotherm, i.e., binding of [^{3}H]oleate at a specific site, superimposed on the nonspecific partitioning of [^{3}H]oleate into the lipids of the plasma membrane. There was no competition between [^{14}C]oleate and [^{3}H]palmitate for entry into plasma membranes. Finally, rates of uptake of [^{14}C]oleate and [^{3}H]palmitate by perfused rat liver were not affected by the presence of the other fatty acid in perfusates. These data indicate that the avidity of hepatocyte plasma membranes for [^{3}H]oleate is a simple consequence of the physical chemical properties of oleate, lipids, and water. The data exclude the idea that the uptake of fatty acids into cells is the result of binding proteins and/or catalyzed reactions at the water–membrane interface of the cell or within the plane of the plasma membrane. — **Cooper, R. B., N. Noy, and D. Zakim.** Mechanism for binding of fatty acids to hepatocyte plasma membranes. *J. Lipid Res.* 1989. **30:** 1719–1726.

Supplementary key words unilamellar lipid vesicles • binding proteins

The prevalent view today is that the entry of fatty acids into cells is catalyzed by one or more specialized proteins (1–8) that facilitate reactions [1] to [4] (below). It was proposed several years ago (9, 10) that fatty acids entered plasma membranes of cells simply as a consequence of their hydrophobicity and then diffused rapidly across these membranes to the inside of cells. More recently, measurements of the spontaneous uncatalyzed rates of reactions [1] to [4] showed that they are far faster than rates of uptake of long chain fatty acids by liver under any conditions (11–17). These observations make it difficult to understand what purpose would be served by putative proteins that facilitate reactions [1] to [4].

$$[1]\quad \text{Alb-Fa} + n\text{H}_2\text{O} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{Alb} + \text{FA} - (\text{H}_2\text{O})_n$$

$$[2]\quad \text{FA} - (\text{H}_2\text{O})_n + \text{PM}_o \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \text{PM}_o - \text{FA} + n\text{H}_2\text{O}$$

$$[3]\quad \text{PM}_o - \text{FA} \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} \text{PM}_i - \text{FA}$$

$$[4]\quad \text{PM}_i - \text{FA} + n\text{H}_2\text{O} \underset{k_2}{\overset{k_{-2}}{\rightleftharpoons}} \text{PM}_i + \text{FA} - (\text{H}_2\text{O})_n$$

PM_o and PM_i are, respectively, the outer and inner halves of the bilayers of the plasma membrane.

Moreover, the partition coefficients for the distribution of fatty acids between membranes, including biological ones, and water are on the order of 10^6 to 10^7 in favor of the membranes, that is $k_2 >> k_{-2}$ (15, 16). These data raise further serious questions about the validity of the proposal that uptake of fatty acids by cells is facilitated by proteins because it is difficult to understand why cells would elaborate proteins to catalyze spontaneous reactions that already are faster than intracellular reactions for the metabolism of fatty acids. The purpose of the experiments in this study was to reexamine the interactions between fatty acids and plasma membranes from hepatocytes. Results from several different experimental approaches to this problem provide no evidence for a membrane-bound fatty acid binding protein.

Abbreviations: ULV, unilamellar lipid vesicles; PL, phospholipid; Alb, albumin.

The data presented below confirm instead that fatty acids enter membranes because of their solubility in the lipid portions of membranes and they validate the conclusion that the transbilayer movement of fatty acids across hepatocyte plasma membranes is a spontaneous, uncatalyzed event.

METHODS

Preparation of liver plasma membranes

Plasma membranes enriched in sinusoidal components were prepared from male Wistar rats, (Charles River breeding labs) weighing 250–300 g, according to the method of Song et al. (18) using differential centrifugation on discontinuous sucrose gradients in bicarbonate buffer at pH 7.0. These membranes were characterized by enzymatic markers as in (18) and were typical of the plasma membrane fraction enriched in the sinusoidal component described therein. Protein was measured by the method of Lowry et al. (19), and phospholipid by the method of Dittmer and Wells (20). Membranes were stored at −80°C until use.

Preparation of lipid vesicles

Unilamellar lipid vesicles (ULVs) were made from egg phosphatidylcholine or whole lipids extracted from plasma membranes (21) by sonication of lipids dispersed in 100 mM KCl, 10 mM Tris, (pH, 7.4) and 1 mM ascorbate. Concentrations of egg phosphatidylcholine and extracted lipid vesicles were 12 μmol/ml and 1–3 μmol/ml of phospholipids, respectively. Vesicles were centrifuged at 100,000 *g* to sediment undispersed material. Known amounts of [^{3}H]oleate (Amersham) were cosonicated with phospholipids for the measurement of distribution coefficients. Vesicles prepared for fusion were cosonicated with 1 mol% myristate and then kept below the phase transition temperature for 4 h (22, 23). The fused vesicles were mixed with small, sonicated vesicles. An aliquot of [^{14}C]myristate was added and the mixture was centrifuged at 100,000 *g* for 15 min. Centrifugation under these conditions results in separation between small vesicles (0.02 μm radius) that remain in the supernatant and larger vesicles that sediment (24). The distribution of [^{14}C]myristate between the supernatant and the pellet was used to calculate the equilibrium distribution of the fatty acid between large and small vesicles.

Measurement of distribution coefficients

The equilibrium distribution of oleate between membranes and albumin or between different populations of membranes represents ratio of the mole fractions of oleate bound to membranes (mole oleate/mole phospholipid) or albumin. These values were determined by measuring the amount of [^{3}H]oleate associated with the albumin or vesicle fractions studied, after separation from plasma membranes (11). In the experiments with plasma membranes and albumin, 50 μl of 0.1 mM [^{14}C]albumin (New England Nuclear) was mixed with plasma membranes containing 200 nmol or phospholipid. [^{3}H]Oleate was added to achieve a range of oleate to albumin ratios (mole/mole) between 0.5 and 4, corresponding to the high affinity binding sites for fatty acids on albumin (25, 26). After incubation at 37°C for 30 min, the mixtures of albumin and membranes or membranes and protein-free vesicles were separated by centrifugation. Data published earlier show that equilibrium distribution of oleate between membranes or between membranes and albumin is achieved within the time of an experiment (11). The time of incubation was far too brief to allow for exchange of significant amounts of lipid between membranes except for oleate (27–30). In addition, aliquots of the supernatants and pellets were counted in two channels for [^{3}H]-oleate and [^{14}C]albumin to assure that there was no sedimentation of albumin. Phosphorus content was determined in the supernatants to control for any failure of the plasma membranes to pellet. In a similar experiment plasma membranes were heated at 70°C for 4 h and then used to determine the distribution of [^{3}H]oleate between heated membranes and albumin (6). Control experiments demonstrated no precipitation of ULVs under conditions used to separate plasma membranes from lipid vesicles or large vesicles from small vesicles, which was confirmed by determination of phosphorus in the appropriate supernatants. The amounts of [^{3}H]oleate dissolved in water in experiments performed in the presence of albumin were negligible as compared with amounts bound to vesicles or albumin because of the high affinity of binding of albumin for fatty acids. The concentration of oleate in water in experiments containing vesicles was always less than 1% of total oleate in the system. This conclusion is based on the partition coefficient of oleate between lipid bilayers and water (ratio of mole fractions) which is about 7×10^6 in favor of the lipid phase (15). The data, therefore, are not corrected for amounts of oleate in water.

Equilibrium distribution of fatty acids between ULVs of egg phosphatidylcholine and hepatocyte plasma membranes was measured in a similar way. Plasma membranes containing about 100 nmol of phospholipids were mixed with vesicles containing the same amount of lipid into which radioactively labeled palmitate or oleate was incorporated by cosonication. In other experiments, fatty acids were added to the assay mixture from ethanol solutions. Results did not vary by varying the method of introduction of fatty acids. After a 30-min incubation at 37°C, membrane components were separated by a 10-min centrifugation in an Eppendorf centrifuge. Supernatants and pellets were counted for the labeled fatty acids. To control for possible fusion or aggregation between vesicles and membranes, plasma membranes were mixed with vesicles containing ^{14}C-labeled dipalmitoyl phosphatidylcholine. Fatty acids were added and the mixture was incubated and centrifuged as described. Essentially

all of the lipids originally associated with the vesicles were found in the supernatant.

Uptake of fatty acids by perfused rat liver

Male Wistar rats were used for the liver perfusion experiments as described by Noy, Donnelly, and Zakim (12). Flow rate of the perfusate was 30 ml/min. After the liver was isolated in situ, it was washed for 5 min with oxygenated Krebs-Henseleit buffer containing 5 mM glucose, 5 mM glutamate, 5 mM pyruvate, and 10 mM HEPES, pH 7.4. Following the stabilization period, the perfusate was exchanged for a buffer containing 0.2 mM bovine serum albumin (Sigma) to which was complexed either 0.1 mM [^{3}H]palmitate or [^{14}C]oleate (New England Nuclear). These solutions were perfused until steady-state uptake was achieved (3–4 min). Aliquots of the effluent were then collected and counted for [^{3}H]palmitate or [^{14}C]oleate to determine the uptake rates of the fatty acids. In experiments where palmitate and oleate were perfused simultaneously, albumin concentration was 0.4 mM and each of the fatty acids was added at a final concentration of 0.1 mM, so that the molar ratio of fatty acid/albumin was kept at 0.5.

RESULTS

Effect of heat treatment on the binding of oleate to plasma membranes

In view of the discrepancies between the arguments for the passive entry of fatty acids into membranes and the proposal that about 50% of fatty acids in plasma membranes from liver is bound to a specific fatty acid binding protein (6), we repeated the experiment on which this conclusion was based (6). It was assumed in reference 6 that heat treatment would destroy the function of a putative binding protein. Thus, Stremmel et al. (6) compared the binding of oleate to untreated and heat-treated plasma membranes. Plasma membranes in **Fig. 1** were heat treated exactly as reference 6, i.e., for 4 h at 70°C. The equilibrium distributions of oleate between albumin and plasma membranes were measured before and after heating (11). Data are given for varying ratios of oleate/albumin (mole/mole) to reflect the variability in the classes of fatty acid binding sites per molecule of albumin, and are plotted as K_{eq} (equation 1) versus the ratio oleate/albumin at equilibrium. PL in equation 1 denotes the amount of phospholipids in the membranes.

$$K_{eq} = \frac{(\text{moles oleate})_{alb}}{\text{moles alb}} \Big/ \frac{(\text{moles oleate})_{PL}}{\text{moles PL}}. \qquad \textit{Eq. 1)}$$

The data in Fig. 1 show that oleate partitioned preferentially to albumin in the albumin–plasma membrane system.

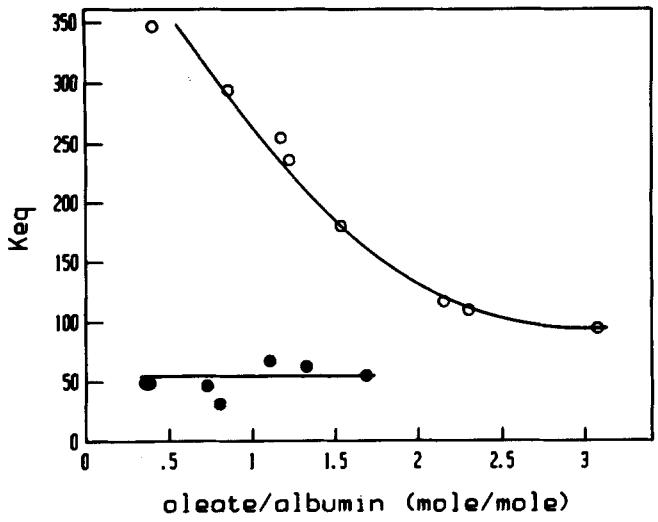

Fig. 1. Effect of heat treatment of plasma membranes on their affinity for [^{3}H]oleate. The partition coefficient for oleic acid was determined between albumin and plasma membranes. Increasing amounts of [^{3}H]oleate were added to 200 nmol of plasma membrane phospholipid and 5 nmol [^{14}C]albumin in a volume of 500 μl. Solid circles represent experiments in which plasma membranes were heated at 70°C for 4 h prior to the addition of oleate and albumin. Membranes were separated from albumin by centrifugation as in Methods. The ratio of the mole fraction of oleate associated with albumin and the mole fraction associated with plasma membranes is expressed as K_{eq}.

Partitioning increasingly favored the plasma membrane as the ratio moles fatty acid/moles albumin increased, which is the expected result since, of the multiple binding sites on albumin, only the first two classes of sites have high avidity for fatty acids (26). The data expressed by open circles, which are for untreated plasma membranes, are comparable to the partitioning of oleate between untreated plasma membranes from rat liver and albumin previously reported (6). The data expressed by closed circles are for partitioning of oleate between albumin and plasma membranes that had been treated at 70°C for 4 h. Contrary to the data in reference 6, heat treatment of plasma membranes increased rather than decreased the amount of oleate partitioning into the membranes. We excluded that the results for heat-treated plasma membranes were due to co-precipitation of albumin–fatty acid complexes with heat-treated membranes.

It is not clear to us why we could not duplicate the results of Stremmel, et al. (6); nor is it clear why heating increased the partitioning of fatty acids into plasma membranes. We will deal first with possible explanations for the basic observation that heating increased partitioning into membranes. It is important in this context to point out that the rationale for Stremmel's experiment (6) was based on the assumption that heating plasma membranes would cause only thermal denaturation of proteins in plasma membranes. No consideration was given to the effects of heat and/or prolonged treatment at 70°C on the activity of proteases and phospho-

lipases. In this sense, the original heating experiments were uncontrolled. We tried, therefore, to determine whether heating could have altered the membranes in ways other than only thermal denaturation of proteins. A possible explanation for enhanced binding of oleate to heat-treated versus untreated plasma membranes in the experiments in Fig. 1 is that heat treatment of the membranes liberated substances that competed with oleate for binding to albumin, which would displace oleate from albumin into the membranes. This possibility was examined by mixing fatty acid-poor albumin with heat-treated plasma membranes in the absence of [^{3}H]oleate. If treatment of plasma membranes produced substances that competed with oleate for binding to albumin, then the albumin in this experiment would have a reduced capacity to bind oleate. Albumin in the above experiment was separated from the plasma membranes by centrifugation, and then added and to untreated plasma membranes. [^{3}H]Oleate was then added and its distribution between albumin and untreated plasma membranes was determined. Partitioning of oleate between this preparation of albumin (exposed to heat-treated plasma membranes) and membranes was identical to that of the control experiment (data not shown)

Other possible explanations for the results depicted in Fig. 1 are that heat treatment caused denaturation of the proteins of the plasma membranes resulting in non-specific binding of oleate to these proteins, or that heat treatment modified the bulk phase properties of the lipid–protein mixture in plasma membranes. We excluded that heat treatment altered the bulk phase properties of the lipids. Thus, partitioning of oleate into unilamellar vesicles comprised of lipids extracted from heat-treated plasma membranes was identical to that for vesicles made from control membranes (data not shown). The reason why treatment at 70°C enhanced partitioning of oleate into plasma membranes was not examined further at this time because the results of such an experiment did not seem relevant to the physiological problem being addressed.

Other experimental tests for defining the basis of the interaction of oleate to plasma membranes

Our inability to reproduce the effect of heating on binding of oleate to plasma membranes is not unexpected in view of the high capacity of lipid bilayers for solubilization of fatty acids in the bulk phase of the membrane. Nevertheless, we pursued the question of the discrepancy between our results and those of Stremmel et al. (6) by three alternate experimental approaches for studying the nature of the interaction between fatty acids and plasma membranes. These were *a*) to measure the distribution of oleate between intact plasma membranes and ULVs comprised of lipids extracted from a separate portion of plasma membranes; *b*) to determine whether binding of oleate to plasma membranes displayed the properties of binding to specific sites (specific binding to a protein) or partitioning into a bulk phase; and *c*) to determine whether two fatty acids competed for binding to plasma membranes. The rationale for the experiment in *a*) was as follows. The lipid composition of the ULVs was identical to that of the plasma membranes. Hence, if the plasma membranes contained fatty acid binding proteins, there would be a greater than 1/1 partitioning of fatty acids into the plasma membranes as compared with the vesicles, the data being normalized on the basis of moles fatty acid/moles lipid in ULVs versus plasma membranes.

Total lipids were extracted from plasma membranes and reconstituted into ULVs by cosonication with [^{3}H]oleate, as in Methods. Vesicles, containing a total of 750 nmol phospholipid and 10 nmol [^{3}H]oleate, were mixed with plasma membranes containing 500 nmol phospholipid. All experiments were done at 37°C. Membrane fractions were separated from each other by centrifugation. The ratio of the mole fraction of oleate in phospholipids of intact plasma membranes to the mole fraction in the membrane phospholipids alone was 0.82. These results indicate that fatty acids did not enter plasma membranes because the latter contained a fatty acid binding protein. In fact, the proteins in plasma membranes decreased the partitioning of fatty acids as compared with membranes that were identical in lipid composition but devoid of protein. The mechanism of this effect was not explored further.

Partitioning of pyrene-containing long chain acyl CoA and acyl carnitine compounds depends on the radius of curvature of the phospholipid vesicles containing these compounds (31). Solubility in this instance increases with increasing radius of curvature. The data for the partitioning of oleate between plasma membranes and lipid vesicles do not suggest that this kind of effect is important for the differential solubility of oleate. Solubility was greater in the population of vesicles with the smaller radius of curvature (i.e., plasma membranes vs ULVs prepared by sonication of lipid dispersed in water). Nevertheless, the possibility that radius of curvature influenced the differential solubility of the fatty acid in lipid bilayers, in any way, was measured directly. Populations of vesicles with identical lipid compositions but different radii of curvature were prepared by allowing unilamellar vesicles of dimyristoyl phosphatidylcholine to fuse with each other (22, 23). The radius of curvature in such vesicles is about fourfold greater than in vesicles of DMPC that have not been allowed to fuse (32). The distribution of [^{14}C]myristate then was determined between large, fused vesicles (950 Å diameter) and small, unfused vesicles (250 Å diameter) after separating the two populations by centrifugation (31, 32). The partition coefficient for the distribution of myristate between large and small vesicles was 1.0, indicating that differences in the radius of curvature cannot account for the differential distribution of oleate between vesicles and plasma membranes.

The rationale for the experiment in *b*) is the idea that the binding of ligand to sites has the properties of a binding iso-

therm. The distribution of a molecule between two bulk phases has the properties of a partition coefficient. Our prior data for the solubility of fatty acids in model membranes (15, 16) predict that the binding of oleate to plasma membranes will have the properties of a partition coefficient. But, if oleate binds to sites on a protein (or proteins) in plasma membranes, then one expects to observe a titration curve as these sites become saturated. To avoid the problem of the exact physical status of oleate added to water (15, 16, 26), plasma membranes were titrated with oleate incorporated into ULVs of egg phosphatidylcholine. One expects to see in such an experiment a titration curve for specific sites superimposed on partitioning of oleate between the lipid region of the plasma membrane and the unilamellar vesicles. A specific binding site should be discernable in the case where a significant amount of total binding of oleate to membranes is due to interaction with a specific protein, as has been suggested (6–8).

The data in **Table 1** show that the distribution of oleate between plasma membranes and ULVs behaved as though oleate in this system distributed between two bulk phases over a 200-fold range of oleate concentrations. Thus, this experimental approach to the problem of how fatty acids interact with plasma membranes also provided no support for the idea that oleate binds to sites in the membrane. On the contrary, these data as well as K_{eq}s for the partitioning of oleate between plasma membranes and protein-free lipid vesicles indicated that oleate interacted with hepatocyte plasma membranes simply because of its solubility in the lipid regions of these membranes.

The last approach [in *c*) above] taken to investigate whether a protein in plasma membranes contributed significantly to binding of fatty acids by these membranes was to test for competition for binding between two fatty acids. If the entry of fatty acids into membranes depends to a detectable extent on the presence of proteins, then fatty acids should compete with each other for binding. The distributions of [^{3}H]palmitate and [^{14}C]oleate between ULVs and plasma membranes were first determined separately. The distributions were then determined for each fatty acid when a mixture of palmitate and oleate was present. **Table 2** summarizes the results from this experiment. It is clear from the data that there was no competition between palmitate and oleate for binding. The distribution of each fatty acid was independent of the presence of the second fatty acid.

TABLE 1. Partitioning of oleate between unilamellar lipid vesicles and intact plasma membranes

Oleate Added to Assay	K_{eq}
nmol	
0.54	0.47
2.14	0.58
4.28	0.55
10.70	0.47
21.40	0.43
53.50	0.43
107.00	0.54

In each experiment plasma membranes containing a total of 150 nmol phospholipids were mixed with 800 nmol of unilamellar vesicles of egg phosphatidylcholine. [^{3}H]Oleate was added as a component of the vesicles of egg phosphatidylcholine. The final volume was 0.50 ml, pH 7.4. Experiments were carried out at 37°C. Membrane components were separated by centrifugation, as in Methods. The equilibrium distributions of oleate are given as K_{eq}, which is the ratio of the mole fraction of oleate in phospholipids of intact plasma membranes to the mole fraction of oleate in the phospholipids of the lipid vesicles. Values are the averages of two experiments.

Rates of uptake of fatty acids by liver perfused simultaneously with two fatty acids

The data presented above show that there is no protein in plasma membranes to facilitate the trapping of fatty acids. These data, however, cannot exclude the presence of trace amounts, e.g., catalytic amounts, of a protein that binds fatty acids during the process of transbilayer movement. In fact, this problem is not accessible by studies of the rate of uptake of fatty acid because this is a process comprised of several discrete steps. The only question that can be addressed by measurements of the rate of uptake is whether any event within the plane of the plasma membrane (i.e., reactions [2] and [3]) are rate determining and are catalyzed by a protein (see Discussion section). If these conditions occur in intact liver, then there will be competition between fatty acids for uptake. This experiment will not depend on events within the cell, whether or not they are catalyzed by protein, because the concentrations of fatty acids in cell water and intracellular membranes are less than the concentrations of proteins to which they bind, as for example the soluble fatty acid binding protein or membrane-bound acyl-CoA ligases (17). The key to the competition experiment, however, is that the concentration of fatty acid in the plasma membranes (oleate for example) of perfused liver is the same for perfusates containing a single type of albumin–fatty acid complex and perfusates containing a second, different complex.

Rat livers were perfused either with [^{14}C]oleate or [^{3}H]palmitate complexed to serum albumin. The ratios of palmitate to albumin and oleate to albumin were 0.5/1 (mol/mol). The dissociation constant for albumin–oleate is fourfold smaller than that for albumin–palmitate (26). Thus, there will be a redistribution of oleate and palmitate among binding sites on albumin when the two complexes are mixed. This was minimized by keeping the ratio of fatty acids/albumin at 0.5/1 (mol/mol). Most important, however, is that the concentration of a given fatty acid in plasma membranes—in a system of membranes, albumin, and water—is not predicted by the dissociation constant for albumin–fatty acid in water, which is k_1/k_{-1}. The amount of fatty acid in the membranes in the above system is given by $(k_1/k_{-1})/(k_2/k_{-2})$. Under the conditions of perfusion we used, K_{eq} (equation 1) for the distribution of palmitate between albumin and hepatic plasma membranes was 205 in the absence of

TABLE 2. Simultaneous partitioning of oleate and palmitate between unilamellar lipid vesicles and intact plasma membranes

Fatty Acid Added		K_{eq}	
Oleate	Palmitate	Oleate	Palmitate
+	–	0.49 ± 0.05[a]	
–	+		0.48 ± 0.03[b]
+	+	0.49 ± 0.03[b]	0.47 ± 0.02[b]

Plasma membranes containing 99.2 nmol of phospholipids were mixed with 100 nmol of unilamellar vesicles of egg phosphatidylcholine. [^{14}C]Oleate (5.18 nmol) or [^{3}H]palmitate (3.78 nmol) or both were added from concentrated ethanol solutions. The final volume was 0.5 ml. After a 30-min incubation at 37°C, membrane components were separated by centrifugation. The equilibrium distribution constants are given as K_{eq}, the ratio of the mole fraction of the fatty acids in the phospholipids of intact plasma membranes to their mole fraction in the phospholipids of the vesicles. Values are given as mean ± SEM.

[a] n = 5.

[b] n = 3.

albumin-oleate and 211 in the presence of albumin-oleate. K_{eq} for the distribution of oleate between albumin and plasma membranes was 200 in the absence of albumin-palmitate and 210 in the presence of albumin-palmitate. The reproducibility of these measurements was + 10%. Therefore, the mixing of albumin-palmitate with albumin-oleate in perfusates did not alter the concentration of either fatty acid in plasma membranes as compared with concentrations of each fatty acid in the absence of the other. A final important point about the conditions for the perfusion experiments was that the concentration of albumin and the ratio of fatty acid to albumin in the perfusates were within the physiologic range. The concentrations of the complexes albumin-fatty acid used for the experiments in **Table 3** also were equal to or greater than reported values of K_m for these complexes for rates of uptake by perfused liver in the steady state (3, 12). The point made by the data in Table 3 is that the uptake of palmitate and oleate proceeded independently.

DISCUSSION

Evidence for the basis of interaction between fatty acids and plasma membranes

The data presented above show that all experimental approaches accessible for the direct study of how fatty acids interact with plasma membranes lead to the conclusion that this interaction is due to the partitioning of fatty acids into the hydrophobic regions of the membranes. Since this conclusion is diametrically opposite to what is apparently the consensus opinion of most workers in the field, which is that uptake of fatty acids into cells is facilitated by a series of proteins that enhance the rates of reactions [1] to [4] (3–8), it is important to review the experimental basis for the latter ideas.

The idea that a plasma membrane protein facilitates reaction [1] (1, 2) has now been abandoned by its original proponent (33). The reasons why such a protein could not contribute to increasing the rate of uptake of fatty acids by liver have been considered elsewhere (12, 17, 34), and we will not discuss it further.

The experimental basis for the notion that proteins can trap fatty acids in membranes (i.e., alter the equilibrium positions of reactions [2] and [4]) is that heating plasma membranes decreased the capacity of these membranes for binding of fatty acids, that a fatty acid binding protein can be purified from plasma membranes of liver, and that an antibody to this protein inhibited the entry of oleate into plasma membranes of liver (6, 35). As shown above, we cannot reproduce the result that heating decreased the binding of fatty acids to plasma membranes. More important than this negative result, several different kinds of experimental approaches failed to demonstrate evidence for binding of detectable amounts of fatty acids to binding proteins. Not only were our experiments more direct than the original heating experiment (6), they also did not depend on assumptions about the effect of heating on the properties of the putative binding protein for fatty acid. This last point may not be trivial. For example, the entry of sulfobromophthalein (BSP) into liver membranes is believed to depend on the same sorts of proteins (35–40) alleged to facilitate the entry of fatty acids into membranes. Yet the laboratory reporting that heat inactivated the binding protein for fatty acids (6) has reported that prior heating of plasma membranes enhanced the subsequent binding of BSP to these membranes (40), which curiously is exactly the result we obtained for the effects of heating on the binding of fatty acids to plasma membranes.

There are inconsistencies in the findings reported from the same laboratory in regard to the idea that oleate enters

TABLE 3. Uptake by the liver of palmitate and oleate when perfused separately and simultaneously

Fatty Acid Perfused		Uptake Rate	
Oleate	Palmitate	Oleate	Palmitate
		nmol/min/g liver	
+	–	9.0 ± 0.86	
–	+		11.1 ± 4.0
+	+	8.7 ± 2.0	13.0 ± 3.9

Livers were perfused with solutions containing 0.1 mM [^{14}C]oleate or [^{3}H]palmitate or both as described in Methods. The concentration of albumin was 0.2 mM in experiments where the fatty acids were perfused separately and 0.4 mM when simultaneous perfusions were performed. When steady-state rates of uptake were established, aliquots of the effluent were collected and analyzed for [^{14}C]oleate and [^{3}H]palmitate. Uptake rates are averages of three perfusions for "separate" experiments and of four perfusions for "mixed" experiments. Values are mean ± SEM.

liver plasma membranes because it binds to a specific binding protein. Thus *i*), as mentioned already, the data in reference 6 allege that heating membranes for 4 h at 70°C decreases the binding of oleate by about 50%, which is the amount of binding attributed therefore to specific binding. By contrast, Stremmel et al. (35) reported that heating membranes for 5 min at 100°C decreased binding by 95%. Although the conditions for heating were not identical in these two reports, the underlying rationale for the heating experiments is that the result provides a measure of the amount of fatty acid bound at specific sites on proteins. *ii*) Specific binding in one study (6) but not in the other (35) was determined on the basis of the results of the heating experiment. *iii*) It was reported (35) that the total of nonspecific and specifically bound oleate reached a limiting value; but the data in (6) do not reproduce this result. *iv*)From the data in (35) one can calculate, on the basis of the methods given in this report, that specific binding was saturated at about 22 nmol oleate/mg membrane protein. In (6), however, specific sites were saturated at 3.2 nmol oleate/mg membrane protein. *v*) Finally, specific binding of oleate to plasma membranes was reported as maximal at 37°C. This result is not compatible with binding at specific sites, which is driven by a negative enthalpy change, but it does fit with a process that is entropy-driven as the partitioning of oleate from water into the hydrophobic interior of the membrane lipids.

Is the transbilayer movement of fatty acids a catalyzed reaction in plasma membranes?

The data in Table 3 show that two different fatty acids do not compete for uptake into the liver. This experiment was carried out using physiologic concentrations of albumin and fatty acids that also equalled or exceeded concentrations that give $V_{max}/2$ of the uptake rate in the steady state (4, 12). The results in Table 3 mean, therefore, that the limiting step in the process by which fatty acids enter cells cannot be catalyzed by a protein within the plasma membrane. The results do not exclude in themselves that the movement of fatty acids across the plasma membranes is catalyzed by a protein at a rate in excess of the steady state rate of uptake. Evidence on this question must come from studies of isolated membranes. The data in this regard make it highly unlikely that transbilayer movement of fatty acids across plasma membranes is catalyzed. For example, in model membrane systems containing saturated acyl chains, k_3 is larger than k_{-2} (11, 13). Both are faster than the steady state rate of uptake of fatty acids by liver (12). The value of k_3 in physiologic lipids (highly unsaturated) will be higher than those measured in model systems (saturated lipids) (cf. ref. 41), and more important, incorporation of proteins into a bilayer will increase k_3 even more (42, 43). Thus, measurements of k_3 in model systems give values that underestimate the rate of reaction [3] in biological membranes. It is difficult to see what purpose could be served by catalyzing the rate of a reaction that already is orders of magnitude faster than the rate-limiting step in the process of uptake of fatty acids by the liver. There is compelling evidence in this regard that the slowest step in this process is intracellular diffusion of fatty acids (17). Whether or not this step is facilitated by a protein remains to be established.

The conclusion that the uptake of fatty acids by the liver is due to a series of spontaneous, uncatalyzed reactions and that the lipid moiety of the plasma membrane and its affinity for fatty acids is the determining factor for the extent to which fatty acids partition into this membrane may point to a possible control mechanism for the process of uptake of fatty acids by cells. Factors that modulate this affinity may be expected to affect uptake rates. Although the detailed nature of these factors is unclear, the solubility of long chain fatty acids in different biological membranes varies to a significant extent (44).

This work was supported in part by the National Institutes of Health (GM-33142). R. B. C. was supported by training grant AM-07142.

Manuscript received 13 March 1989 and in revised form 19 June 1989.

REFERENCES

1. Weisiger, R. A., J. L. Gollan, and R. K. Ockner. 1981. Receptor for albumin on the liver cell surface may mediate uptake of fatty acids and other albumin-bound substances. *Science.* **211:** 1048–1051.
2. Ockner, R. K., R. A. Weisiger, and J. L. Gollan. 1983. Hepatic uptake of albumin-bound sustances: albumin receptor concept. *Am. J. Physiol.* **245:** G13–18.
3. Weisiger, R. A. 1985. Dissociation from albumin: potentially rate-limiting step in the clearance of substances by the liver. *Proc. Natl. Acad. Sci. USA.* **82:** 1563–1567.
4. Abumrad, N. A., R. C. Perkins, J. H. Park, and C. R. Park. 1981. Mechanism of long chain fatty acid permeation in the isolated adipocytes. *J. Biol. Chem.* **256:** 9183–9191.
5. Abumrad, N. A., C. R. Park, and R. R. Whitesell. 1986. Catecholamine activation of the membrane transport of long chain fatty acids in adipocytes is mediated by cyclic AMP and protein kinase. *J. Biol. Chem.* **261:** 13082–13086.
6. Stremmel, W., G. Strohmeyer, F. Borchard, S. Kochwa, and P. D. Berk. 1985. Isolation and partial characterization of a fatty acid binding protein in rat liver plasma membranes. *Proc. Natl. Acad. Sci. USA.* **82:** 4–8.
7. Stremmel, W. 1987. Translocation of fatty acids across the basolateral rat liver plasma membrane is driven by an active potential-sensitive sodium-dependent transport system. *J. Biol. Chem.* **262:** 6284–6289.
8. Stremmel, W., and P. D. Berk. 1986. Hepatocellular influx of [^{14}C]oleate reflects membrane transport rather than intracellular metabolism or binding. *Proc. Natl. Acad. Sci. USA.* **83:** 3086–3090.
9. Spector, A. A., D. Steinberg, and A. Tanaka. 1965. Uptake of free fatty acids by Ehrlich ascites tumor cells. *J. Biol. Chem.* **240:** 1032–1041.
10. De Grella, R. F., and R. J. Light. 1980. Uptake and metabolism of fatty acids by dispersed adult rat heart myocytes. II.

Inhibition by albumin and fatty acid homologues, and the effect of temperature and metabolic reagents. *J. Biol. Chem.* **255:** 9731–9738.
11. Daniels, C., N. Noy, and D. Zakim. 1985. Rates of hydration of fatty acids bound to unilamellar vesicles of phosphatidylcholine or to albumin. *Biochemistry.* **24:** 3286–3292.
12. Noy, N., T. M. Donnelly, and D. Zakim. 1986. Physicalchemical model for the entry of water-insoluble compounds into cells. Studies of fatty acid uptake by the liver. *Biochemistry.* **25:** 2013–2021.
13. Doody, M. C., H. J. Pownall, Y. J. Kao, and L. C. Smith. 1980. Mechanism and kinetics of transfer of a fluorescent fatty acid between single-walled phosphatidylcholine vesicles. *Biochemistry.* **19:** 108–116.
14. Hamilton, J. A., and D. P. cistola. 1986. Transfer of oleic acid between albumin and phospholipid vesicles. *Proc. Natl. Acad. Sci. USA.* **83:** 82–86.
15. Noy, N., and D. Zakim. 1985. Fatty acids bound to unilamellar vesicles of phosphatidylcholine as substrates of microsomal fatty-acyl-CoA ligase. *Biochemistry.* **24:** 3521–3525.
16. Noy, N., and D. Zakim. 1985. Substrate specificity of fatty-acyl-CoA ligase in liver microsomes. *Biochim. Biophys. Acta.* **833:** 239–244.
17. Cooper, R. B., N. Noy, and D. Zakim. 1987. A physical-chemical model for cellular uptake of fatty acids: prediction of intracellular pool sizes. *Biochemistry.* **26:** 5890–5896.
18. Song, C. S., W. Rubin, A. B. Rifkind, and A. Kappas. 1969. Plasma membranes from rat liver. Isolation and enzymatic characterization of a fraction rich in bile canaliculi. *J. Cell Biol.* **41:** 124–132.
19. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:** 265–275.
20. Dittmer, J. C., and M. A. Wells. 1969. Quantitative and qualitative analysis of lipids and lipid components. *Methods Enzymol.* **14:** 482–530.
21. Kates, M. 1972. Techniques in Lipidology. North Holland Publishing co., Amersterdam, London. 32.
22. Kantor, H. L., and J. H. Prestegard. 1978. Fusion of phosphatidylcholine bilayer vesicles: role of free fatty acid. *Biochemistry.* **17:** 3592–3597.
23. Scotto, A., and D. Zakim. 1985. Reconstitution of membrane proteins. Spontaneous association of integral membrane proteins with preformed unilamellar vesicles. *Biochemistry.* **24:** 4066- 4075.
24. Barenholz, Y., D. Gibbs, B. J. Litman, J. Goll, T. E. Thompson, and F. D. Carlson. 1977. A simple method for the preparation of homogeneous phospholipid vesicles. *Biochemistry.* **16:** 2806–2810.
25. Hamilton, J. A., D. P. Cistola, J. D. Morrisett, J. T. Sparrow, and D. M. Small. 1984. Interactions of myristic acid with bovine serum albumin: a ^{13}C NMR study. *Proc. Natl. Acad. Sci. USA.* **81:** 3718–3722.
26. Specor, A. A. 1975. Fatty acid binding to plasma albumin. *J. Lipid Res.* **16:** 165–179.
27. Massey, J. B., A. M. Gotto, Jr., and H. J. Pownall. 1982. Kinetics and mechanism of the spontaneous transfer of fluorescent phosphatidylcholines between apolipoprotein-phospholipid recombinants. *Biochemistry.* **21:** 3630–3636.
28. McLean, L. R., and M. C. Phillips. 1981. Mechanism of cholesterol and phosphatidylcholine exchange or transfer between unilamellar vesicles. *Biochemistry.* **20:** 2943–2900.
29. Petsry, G. E., and A. Jonas. 1984. Spontaneous phosphatidylcholine exchange between small unilamellar vesicles and lipid-apolipoprotein complexes. Effects of particle concentrations and compositions. *Biochemistry.* **23:** 720–725.
30. Wolkowitz, P. E., H. J. Pownall, D. F. Pauly, and J. B. McMillin-Wood. 1984. Pyrenedodecanoylcarnitine and pyrenedodecanoyl coenzyme A: kinetics and thermodynamics of their intermembrane transfer. *Biochemistry.* **23:** 6426–6432.
31. Szoka, F., Jr., and D. Papahadjopoulos. 1980. Comparative properties and methods of preparation of lipid vesicles (liposomes). *Annu. Rev. Biophys. Bioeng.* **9:** 467–508.
32. Wong, M., and T. E. Thompson. 1982. Aggregation of dipalmitoyl phosphatidylcholine vesicles. *Biochemistry.* **21:** 4133–4139.
33. Weisiger, R. A., and W-L. Ma. 1987. Uptake of oleate from albumin solutions by rat liver. Failure to detect catalysis of the dissociation of oleate from albumin by an albumin receptor. *J. Clin. Invest.* **79:** 1070–1077.
34. Zakim, D., N. Noy, and R. B. Cooper. 1988. Uptake of water-insoluble compounds by tissues. *In* Molecular Basis of Drug Action. T. P. Singer, N. Castagnoli, and C. C. Wang, editors. Walter de Gruyter, New York. 35–48.
35. Stremmel, W., M. A. Gerber, V. Glezerov, S. N. Thung, S. Kochwa, and P. D. Berk. 1983. Physiochemical and immunohistological studies of sulfobromophthalein- and bilirubin-binding protein from rat liver plasma membranes. *J. Clin. Invest.* **71:** 1796–1805.
36. Stremmel, W., and P. D. Berk. 1986. Hepatocellular uptake of sulfobromophthalein (BSP) and bilirubin is selectively inhibited by an antibody to the liver plasma membrane BSP/bilirubin binding protein. *J. Clin. Invest.* **78:** 822–825.
37. Lunazzi, G., C. Tiribelli, B. Gazzin, and G. L. Sottocasa. 1982. Further studies on bilitranslocase, a membrane protein involved in hepatic organic anion uptake. *Biochim. Biophys. Acta.* **685:** 117–122.
38. Sottocasa, G. L., G. Baldini, G. Sandri, G. Lunazzi, and C. Tiribelli. 1982. Reconstitution in vitro of sulfobromophthalein transport by bilitranslocase. *Biochim. Biophys. Acta.* **685:** 123–128.
39. Berk, P. D., B. J. Potter, and W. Stremmel. 1987. Role of plasma membrane ligand-binding proteins in the hepatocellular uptake of albumin-bound organic anions. *Hepatology.* **7:** 165–176.
40. Reichen, J., B. L. Blitzer, and P. D. Berk. 1986. Binding of unconjugated and conjugated sulfobromophthalein to rat liver plasma membrane fractions in vitro. *Biochim. Biophys. Acta.* **640:** 298–312.
41. Homan, R., and H. J. Pownall. 1987. Effect of pressure on phospholipid translocation in lipid bilayers. *J. Am. Chem. Soc.* **109:** 4759–4760.
42. Van der Steen, A. T. M., W. A. C. De Jong, B. De Kruijiff, and L. L. M. Van Deenen. 1981. Lipid dependence of glycophorin-induced transbilayer movement of lysophosphatidylcholine in large unilamellar vesicles. *Biochim. Biophys. Acta.* **647:** 63–72.
43. Greenhut, S. F., and M. A. Roseman. 1985. Cytochrome b_5-induced flip-flop of phospholipids in sonicated vesicles. *Biochemistry.* **24:** 1252–1260.
44. Cooper, R. B., N. Noy, and D. Zakim. 1986. A physical-chemical model for hepatic uptake of fatty acids. *Hepatology.* **6:** 1187.