Mechanism of Long Chain Fatty Acid Permeation in the Isolated Adipocyte*  

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(Received for publication, April 23, 1981)

The mechanism of permeation of [14C]oleate into isolated rat adipocytes has been examined. The initial rates of uptake of the fatty acid were determined at 23 °C as a function of the concentration of unbound fatty acid in the medium. Under the conditions employed, the following observations were made. 1) The rates were maximal and linear for at least 15 s and were the same in the presence or absence of glucose. 2) In the absence of glucose, all fatty acid taken up was recovered in the cell as unesterified fatty acid, whereas about 75% was esterified in the presence of the sugar. Thus uptake appeared to be independent of fatty acid metabolism. 3) Influx of fatty acid was strongly inhibited by phloretin, particularly at low concentrations of unbound fatty acid. Efflux was also blocked by phloretin in an albumin-free medium at 0 °C was a very effective stop solution for abruptly terminating fatty acid fluxes and for washing cells without loss of unesterified fatty acid. 4) The fatty acid taken up was not bound to the cell surface but probably was largely bound within the cell. 5) Uptake was not limited by dissociation of fatty acid from albumin in the medium nor by an interaction between albumin and the cell surface. From these considerations, we concluded that the uptake measurements were valid estimates of the influx of fatty acid. Partial saturation was observed as a function of external concentration of unbound fatty acid with a k₉₅ of 6 × 10⁻⁸ M.

It appears that permeation by long chain fatty acids is facilitated by a saturable, phloretin-inhibitable mechanism that is presumably protein in nature. Permeation is almost entirely by this mechanism at physiological levels of unbound fatty acid. At high concentrations of unbound fatty acid, however, permeation by a mechanism having diffusion kinetics is detectable, which may indicate some limited passage of fatty acid directly through the phospholipid bilayer.

Passage of most metabolites through the plasma membrane of mammalian cells is by specialized transport systems that are often important points of metabolic control. Long chain fatty acids are quantitatively the most important substrates for energy production and their metabolism is highly regulated. It is therefore a significant question to determine how these substances permeate the cell.

We have attempted to characterize the permeation process in the adipocyte, which is a major site for uptake and release of fatty acids. Metabolism of fatty acids in this cell is profoundly influenced by hormones, as shown in many in vivo and in vitro studies.

Earlier work on fatty acid uptake by adipose tissue was carried out using whole tissue incubations in media containing fatty acids bound to serum albumin. Uptake was related to the total fatty acid concentration in the medium without taking into account the fatty acid interaction with its carrier (1). From studies on the distribution of fatty acids between heptane and albumin containing buffer, Goodman (2) in 1958 showed that the unbound, free fatty acid concentration varied as a function of the ratio of fatty acid to protein. These observations were expanded by Spector et al. (3), who demonstrated the presence of up to 6 physiologically relevant binding sites for fatty acids on albumin.

The cellular uptake of fatty acids was then shown to depend on the fatty acid:BSA ratio by Shtacher and Shafir (4), Steinberg (5) and, in more detail, by Spector et al. (6) using Ehrlich ascites tumor cells. The latter authors observed an initial rapid uptake that was a roughly linear function of the fatty acid:BSA ratio. They concluded, as had Goodman earlier in studies with erythrocytes (7), that fatty acid was first adsorbed to cellular binding sites from which it was then transferred into the metabolic pool by unspecified mechanisms. Very recently, Weisiger et al. (8), working with a perfused liver preparation, reiterated these earlier suggestions and postulated that fatty acid transfer into the cell was dependent on the binding of the fatty acid-albumin complex to the cell surface (8). Other recent studies have considered the permeation process more specifically and a protein carrier facilitating fatty acid entry has been suggested. Mahadevan and Sauer (9), working with isolated hepatocytes, have shown that α-bromopalmitate, trypsin, and phospholipases A and C inhibit oxidation of palmitate under conditions which suggest involvement of a fatty acid transporter in the plasma membrane. Samuel et al. (10) and Paris et al. (11) also have suggestive evidence for a saturable fatty acid uptake process in embryonic chick cardiac cells. Odom (12) found linoleate uptake to be a saturable trypsin-sensitive process in isolated rat adipocytes. On the other hand, DeGrella and Light (13, 14) recently concluded that fatty acids penetrate adult rat heart myocytes by simple diffusion and that the saturable uptake component observed in previous studies reflected predominantly metabolism. Against this latter interpretation, however, are the observations that fatty acid diffusion through black, phospholipid membranes (11) and the “flip-flop” of
fatty acids across the two leaflets of the plasma membrane are very slow processes compared to cellular fatty acid uptake (15). In our view, these studies have not considered adequately the numerous special problems which are involved in fatty acid permeation. As a consequence, there is still no clear answer as to how fatty acid enters a cell. In the present paper, we discuss these special problems and describe how we have attempted to solve them.

**EXPERIMENTAL PROCEDURES**

**Isolation of Fat Cells**—Adipocytes were prepared from the epididymal fat pads of two 200- to 250-g Sprague-Dawley rats (Harlan Industries, Indianapolis, Ind.) fed ad libitum. The fat tissue was digested for 30 min at 37°C with 2 mg/ml of crude collagenase (Worthington Biochemical Corp., Freehold, N. J.) The digestion medium was a Krebs-Ringer medium lacking phosphate and buffered with Heps buffer, pH 7.4, containing 2% BSA (Fraction V, fatty acid-free, Sigma, St. Louis, Mo.) with or without 2 mM glucose, as specified. The cells were obtained by filtration through coarse nylon mesh; they were washed once with Krebs-Ringer medium lacking phosphate and buffered with Heps buffer containing 2 mM glucose, and the suspension was stirred by gentle aspiration, and 50-ml filter (Gelman type A/E 61630, Gelman, Ann Arbor, Mich.) at a rate of 50 ml/min. The filters were prewetted with 1 ml of aqueous counting solution (ACS, Amersham, Arlington Heights, Ill.) and the vials were counted in a Beckman LS 1800 liquid scintillation counter (Beckman Instruments, Fullerton, Calif.). The recovery of cells was found to depend on the fatty acid/BSA ratio and unbound fatty acid concentration. It was very reproducible, however, for any given experimental circumstance and constituted about 15% of the 15-s uptake values at a fatty acid/BSA ratio of 0.5, and 25% at ratios of 3 and above. The fatty acid that adsorbed to the filters without cells was routinely checked, and subtracted from values measured with the cells. Attempts to decrease the background adsorption through the use of different types of filter or prewashing solutions were not successful.

**Preparation of Albumin-bound Fatty Acids**—A stock of albumin-bound fatty acid was prepared by dissolving labeled and unlabeled fatty acid Na salts (fatty acid:NaOH, 1:1) in water at 40°C to give a concentration of about 60 μM. When the solution was completely clear, BSA from a concentrated stock (20%) was added with gentle mixing to give a concentration of 11 μM and a fatty acid:BSA molar ratio of 0.5. Aliquots were frozen at −20°C until use for the assay, an aliquot of the stock was diluted by one-half volume of Krebs-Ringer buffer (1.5 times) made up with or without additional fatty acid-free albumin to obtain the desired concentration and ratio to protein. Stock solutions of albumin-bound fatty acid were also prepared by adding the fatty acid in ethanol with vigorous stirring directly to calcium-free Krebs-Ringer buffer. The final concentration of fatty acid was usually 40 μM. BSA and Ca²⁺ were added at reduced stirring speed to achieve the desired Ca²⁺ concentration (1 mm) and fatty acid:BSA molar ratio. Further details will be given elsewhere.

**Separation of Cellular Lipids**—When incorporation of [¹⁴C]oleate into the plasma lipid was found to be checked, Millipore filters resistant to organic solvents were used (SCPW, Millipore Corp., Bedford, Mass.). Filters plus cells were extracted twice in 4 ml of chloroform/methanol (2:1). The extracts were pooled and 0.2 volume of water was added to separate out as aqueous phase. An aliquot (4 ml) of the chloroform phase was evaporated under a stream of nitrogen and then redissolved in 200 μl chloroform. Twenty-μl samples were then streaked on silica gel plates (Applied Science, Inglewood, Calif.). The developing solvent was petroleum ether/diethyl ether/acetic acid (86:14). The bands were visualized by exposure to iodine vapors and identified by comparison to standards that were run simultaneously. Radioactivity in the bands was counted in a toluene-based scintillation fluid (Liquifluor, New England Nuclear, Boston, Mass.) A correction was made for counts trapped in the extracellular volume and adsorbed to the filters. This was obtained from a zero time value where the cell aliquot was added to already mixed assay medium and stop solution, and then immediately filtered and washed.

**Phloretin Competition for Fatty Acid Binding to BSA**—Phloretin competition for fatty acid binding to BSA was assessed using EPR and the fatty acid analogue 16-nitroxyl stearate (Syva, Palo Alto, Calif.), according to a procedure to be described in detail elsewhere. The fatty acid analogue was added with stirring to ethanol (1 ml) before use.

**Calculations**—(a) Calculation of unbound and bound fatty acid distribution for any particular fatty acid/BSA ratio was carried out as follows. We developed an algorithm based on the model of stepwise equilibrium development by Klotz et al. (16) utilizing the association constant given by Spector (17). Briefly, the allowed reactions are of the form

$$\text{FA} + \text{BSA} \leftrightarrow \text{FA-BSA}$$

where $\text{P}$ is protein, $\text{A}$ is ligand, and $K_{\text{FA-BSA}}$ is the association constant for the formation of the $i + 1$ complex. The reactions of Equation 1 give rise to the equilibrium expression:

$$[\text{FA}]_i + [\text{BSA}]_i = [\text{FA-BSA}]_i [\text{FA}]_i [\text{BSA}]_i$$

Equation 3 was used to estimate free protein and free acid given $A$s. An initial guess was made for $A$, and the value of the right side of Equation 3 was compared to $A$. $A$ was adjusted until it agreed with the input ($A$s) within 0.1% of $A$. Bound ligand distribution was then estimated using Equation 2. The distribution of bound and unbound fatty acid obtained agreed with values obtained using previously published similar computer routines (18). In addition, computation of unbound fatty acid concentrations, using the association constant of the spin label fatty acid 16 nitroxyl stearate gave estimates which were in agreement with experimentally measured values.

(6) The instantaneous concentration of free fatty acid during uptake was calculated on the basis of the following reactions:

1. The abbreviations used are: Heps, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; BSA, bovine serum albumin; KRH, Krebs-Ringer medium buffered with Heps; 16-nitroxyl stearate, 2,14-carboxybetadecyl-2-ethyl-4,4-dimethyl-3-oxazolidinylxyl.

2. R. C. Perkins, N. A. Abumrad, J. H. Park, and C. R. Park, manuscript to be submitted for publication; available on request.
where ubFA is unbound fatty acid, FA is fatty acid, and \( K_a \), \( K_d \), and \( K_e \) are the association rate constant, dissociation rate constant, and uptake rate constant, respectively. These reactions give rise to a set of coupled differential equations.

\[
\frac{dx}{dt} = K_d (B_0 - x) - K_{xy} \tag{6}
\]

\[
\frac{dy}{dt} = K_d (B_0 - x) - K_{xy} - K_{xy} \tag{7}
\]

\[
\frac{dz}{dt} = K_{xy} \tag{8}
\]

where \( x \), \( y \), and \( z \) are the instantaneous values of free BSA and unbound and cell-associated fatty acid, respectively. \( B_0 \) is the total BSA concentration. Equation 7 was solved by a Runge-Kutta algorithm developed by Dr. Philip Crooke, from the Department of Mathematics, Vanderbilt University. The value of \( K_a \) was that for oleate and human serum albumin (19). \( K_e \) was estimated from the association constants of Spector (17) for oleate-BSA complexes, and \( K_e \) was derived from our uptake data.

**RESULTS**

**Nature of the Problem**—Determination of the initial rate of uptake of the substance as a function of its external concentration can distinguish simple diffusion (a linear relationship) from transport (a saturable process). Initial rates are the predominantly rate-limiting step for uptake, and second, that the rates observed are initial rates. It is particularly difficult to satisfy these criteria in the case of fatty acid uptake for several reasons, as will be considered below.

**Measurement of Uptake**—For uptake measurement to be reliable, radioactivity must not be lost from the cell during uptake, cell separation, and washing procedures.

We have measured initial rates of uptake by adipocytes over a period of only 5 s at 23 °C, during which time no metabolites of \(^{14}\)C-labeled fatty acids are released from the cell. A substantial lag in CO\(_2\) release from \(^{14}\)C-labeled fatty acids in hepatocytes has been observed by Mahadevan and Sauer (9). Odom (12) could detect no labeled CO\(_2\) from \(^{1-14}\)C linoleate taken up by adipocytes over a 2-min interval at 37 °C.

Uptake was interrupted abruptly and loss of intracellular counts were largely prevented during separation of cells from the medium and washing (see “Experimental Procedures”) by the use of a stop solution. To provide a critical test of its effectiveness in inhibiting efflux of fatty acids, we first raised the content of unesterified fatty acids in the adipocyte as follows.

Adipocytes were isolated and maintained at room temperature in the absence of glucose in order to reduce esterification. As seen in Fig. 1A, almost all the \(^{14}\)C-olaate taken up by these cells in 30 s was recovered in unesterified form and less than 8% was esterified. The addition of phloretin, a known inhibitor of glucose (20), \( \beta \)-hydroxybutyrate (21), and other transport systems (22, 23), to the incubation medium resulted in a 50% reduction of \(^{14}\)C-olaate uptake. The fatty acid was again recovered in the unesterified form. The absence of esterification held true over a wide range of unbound fatty acid concentrations in the incubation medium (Fig. 1B).

Using cells preloaded with unesterified \(^{14}\)C-olaate, methods to inhibit efflux were then explored (Fig. 2). It is apparent that almost all intracellular fatty acid was released rapidly into buffer containing 1% BSA or into buffer containing unlabelled oleate and 0.25% BSA, even at near 0 °C. The completeness of the washout confirmed that no fatty acid was esterified under these conditions. A substantial efflux of fatty acid (about 50%) also occurred when the cells were diluted with cold buffer alone. In this case, the virtual cessation of efflux

**Fig. 1. Incorporation of \(^{14}\)C-olaate into cellular lipids. Fatty acid (FA) uptake was measured at 23 °C (see “Experimental Procedures”). The filters with the cells were immediately immersed in 4 ml of chloroform/methanol (2:1). Lipids were separated on silica gel plates with petroleum ether/diethyl ether/acetic acid (80:20:1). The counts recovered in the fatty acid band were corrected for adsorption to the filters and trapping in the extracellular medium surrounding the cells. A, time course of \(^{14}\)C-olaate incorporation in the different lipids at a fatty acid:BSA ratio of 0.35: unbound fatty acid = 0.048 \( \mu \)M. B, cellular fate of \(^{14}\)C-olaate at different unbound fatty acid concentrations (fatty acid:BSA ratio from 0.5 to 4), calculated as described under “Experimental Procedures.” The incubation time was 30 s and the total fatty acid concentration was 20 \( \mu \)M.

**Fig. 2. Efflux of \(^{14}\)C-olaate. Adipocytes (30-\( \mu \)l cell suspension) were incubated at 23 °C for 15 s with 15 \( \mu \)l of assay medium containing \(^{14}\)C-olaate (1 \( \mu \)Ci/ml). At zero time, 1 ml of the specified buffer was added. At the times shown on the ordinate, 5 ml of chilled stop solution containing 400 \( \mu \)M phloretin were added and the mixture was filtered. Controls corresponding to 100% radioactivity were cells to which 5 ml of cold stop solution were added at the end of the 15-s incubation period. Total fatty acid was 20 \( \mu \)M. The unbound fatty acid concentration was 0.043 or 0.42 \( \mu \)M, corresponding to fatty acid:BSA ratios of 0.5 and 2, respectively. Points are averages of 4 observations.
after 5 min, despite residual unesterified fatty acid, may have been due to limited solubility of the fatty acid in the buffer, or to an equilibrium partitioning of fatty acid between cells and medium. Increasing the volume of diluting buffer to 10 ml of ice-cold buffer containing the specified phloretin concentration was added and the mixture was then filtered at the times shown on the ordinate. Points are means of duplicates from 2 different experiments using cells loaded with fatty acid by preliminary exposure to either 0.943 or 0.42 μm unbound fatty acid.

It is apparent from the above that stopping uptake of fatty acid and/or washing cells by dilution with a large volume of cold buffer (12) with or without added albumin or unlabeled fatty acid (10, 11) could result in a large loss of unesterified fatty acid and underestimation of uptake.

Cold albumin-free buffer containing 200 μM phloretin stopped efflux effectively (Fig. 2). Negligible loss of 14C-labeled fatty acid occurred in the time required to filter and wash the cells (approximately 15 s), as shown in Fig. 3. Maximum inhibition appeared to be reached with a phloretin concentration of 200 μM. Albumin was omitted from the stop solution, since it was found in separate experiments to diminish the effectiveness of phloretin. However, there was some albumin carried over from the medium used for uptake assay in amounts that depended on the ratio of fatty acid to BSA, but the final concentration of BSA after dilution with the stop medium was less than 0.05% in all cases. As much as 0.1% BSA could be tolerated without impairing significantly the inhibition of efflux.

The stopping procedure was very effective in preventing influx under the conditions of our uptake measurements. We found, for example, that the radioactivity recovered was about the same when a mixture of labeled medium and stop solution was filtered with or without addition of a standard aliquot of cells immediately or up to 2 min before filtration. This result also showed that the presence of cells did not increase 14C-labeled fatty acid trapping in the fluid that remained on the filter. This was confirmed by the use of tritiated insulin and sorbitol in some assays.

Phloretin and Cell Integrity—The concentration of phloretin used in the stop solution did not cause cell lysis, as demonstrated in the experiments of Fig. 4. Adipocytes preincubated with labeled [3H]-2-deoxyglucose did not lose radioactivity (presumably as [3H]-2-deoxyglucose 6-phosphate) when exposed to phloretin at concentrations up to 500 μM. A concentration of 800 μM caused appreciable lysis.

Concentration of Unbound Fatty Acids in the Medium—In order to measure fatty acid uptake as a function of the concentration of unbound fatty acid it is necessary to know that the dissociation of the fatty acid-BSA complex was rapid enough relative to uptake by the cells. No method for the direct estimate of unbound fatty acid concentration during uptake is available, but recently, the dissociation rate constants for human albumin-oleate complexes have been estimated by Scheider (19). Since the binding of fatty acid to human albumin is somewhat tighter than to bovine serum, the dissociation rate constant of 0.2 s⁻¹ was used for the calculations. The dissociation rate constant of 0.2 s⁻¹ was used for the calculations. The dissociation rate constant of 0.2 s⁻¹ was used for the calculations. The dissociation rate constant of 0.2 s⁻¹ was used for the calculations. The dissociation rate constant of 0.2 s⁻¹ was used for the calculations.
albumin (17), the dissociation rate constant for the bovine complex is presumably faster. This derives from the finding that association is diffusion-limited (19).

On this basis, we have calculated the change in concentration of unbound fatty acid in the medium during an uptake assay. The conditions have been selected to correspond to circumstances under which a fall in unbound fatty acid concentration would be most likely, i.e. when the fatty acid:BSA is very low.

The results of the above calculations (see “Experimental Procedures”) are shown in Fig. 5 for an uptake assay under our usual conditions and a fatty acid:BSA ratio of 0.2. At this ratio, which is below any employed in our studies, about 90% of the fatty acid is bound to the highest affinity site. The uptake was assumed to proceed at the initial (i.e. maximal) rate and is plotted linearly up to 60 s on the figure. As can be seen, the calculation indicates that the fall in unbound fatty acid concentration is insignificant during this time interval. Any changes in unbound fatty acid concentration at higher fatty acid:BSA ratios would be expected to be less.

Stability of Cell Preparations—The uptake rates measured in duplicate or triplicate were very reproducible. Fig. 6 shows the stability of uptake in adipocytes isolated and maintained in the absence of glucose. The rates were stable for at least 60 min. Similar observations were made in cells isolated and maintained in 2 mM glucose.

Initial Rate of Oleate Uptake—The uptake of oleate with time by adipocytes isolated and maintained without glucose is shown in Fig. 7. Three concentrations of unbound fatty acid were employed, ranging from 0.04 to 0.4 μM. The uptake under these conditions did not involve metabolism, as shown by the experiments of Fig. 1, and was due therefore either to adsorption or permeation. It is convenient at this point to discuss uptake as the latter process, and we will justify this approach shortly.

During the first 15 s, the uptake in each case was nearly linear with time and thus approximated a true initial rate. That is to say, the rate reflected influx as a function of external concentration during an interval of time before measurable efflux could develop as a result of intracellular accumulation.

The effects of glucose and phloretin on fatty acid uptake with time are shown in Fig. 8. Glucose had little effect on uptake compared to the control rate during the first 15 s, but thereafter maintained uptake at the initial rate, whereas the control rate declined. Phloretin was strongly inhibitory.

Fatty Acid Uptake as a Function of Unbound Fatty Acid Concentration—Using conditions which allow initial rate measurements, the uptake of oleate as a function of unbound fatty acid concentration in the medium appeared to be a saturable process (Fig. 9). A Lineweaver-Burk plot of the data (not shown) provided a k_{ass} value of about 6 x 10^{-8} M. At all unbound fatty acid concentrations in the absence of glucose, fatty acid uptake was accounted for quantitatively by accumulation of unesterified fatty acid in the cell (Fig. 9). In the presence of glucose, the initial rates of uptake were the same as those of the control, but about 75% of uptake was recovered as triglyceride and phospholipid esters. This experiment demonstrates that the initial rate of uptake is independent of metabolism. It also suggests strongly that saturation does not

5This follows from consideration that the equilibrium association constant decreases at higher fatty acid:BSA ratios whereas the association rate constant remains diffusion-limited (19). Thus, the dissociation rate constant must increase. As the unbound fatty acid concentration is raised, uptake increases proportionately or to a lesser degree. Thus, the same or a smaller fraction of unbound fatty acid in the medium will be removed per unit time as the concentration of unbound fatty acid increases.

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![Fig. 6](image6.png)

**Fig. 6. Stability of the adipocyte preparation.** Fatty acid uptake rates are shown in adipocytes isolated in glucose-free medium and maintained at 23°C as a 40% suspension in KRH buffer without glucose and containing fatty acid (20 μM) and BSA (40 μM). ○, cells assayed immediately after isolation; ●, same cell preparation assayed 60 min later.

![Fig. 7](image7.png)

**Fig. 7. Time course of [14C]oleate uptake.** Uptake was measured at 23°C at 3 concentrations of unbound fatty acid (μBFA) in the medium. These were obtained by varying the fatty acid:BSA ratio in the incubation medium from 0.5 to 2.0 at a constant total fatty acid concentration of 20 μM. Calculations and procedures are given under “Experimental Procedures.” Uptake is expressed as nanomoles of fatty acid per 20 μl of packed cells (as contained in 50 μl of a 40% cell suspension). 20 μl of packed cells contain about 4 mg of lipid. Points are means of duplicates from 3 different experiments.

![Fig. 8](image8.png)

**Fig. 8. Effect of glucose and phloretin on [14C]oleate uptake by adipocytes.** Uptake was measured at 23°C in medium containing 0.068 μM unbound fatty acid (obtained by addition of 20 μM oleate and 29 μM BSA). Phloretin or glucose were added only to the assay medium. Points are averages of triplicates from 2 experiments.

reflect filling of binding sites for fatty acid, since the rates are the same despite a great difference in accumulation of unesterified fatty acid. These points will be discussed more fully below.
The experiment was repeated without glucose but the concentration of unbound fatty acid was greatly increased (Fig. 10). A nonsaturable component of uptake now appeared. The estimated contribution of this (linear) component to uptake is shown by the broken line labeled diffusion, whereas the contribution of the saturable component alone is labeled transport.

Effect of Phloretin—The effect of phloretin on oleate uptake in media containing increasing ratios of fatty acid to BSA is shown in Fig. 11. The compound was inhibitory at low ratios but appeared to be stimulatory at higher ratios. This anomalous behavior could be explained in part by the finding that phloretin raised the concentration of unbound fatty acid in the medium by displacing fatty acid from the low affinity sites on BSA. The latter are occupied to an increasing degree as the fatty acid:BSA ratio is raised.

This displacement could be shown (Table I) by electron paramagnetic resonance spectroscopy employing the spin-labeled fatty acid analog 16-nitroxyl stearate. In a separate study, it has been shown that EPR spectroscopy distinguishes quantitatively between bound and free spin-labeled fatty acid in mixtures of both. 16-Nitroxyl stearate was also shown to be a satisfactory analogue of oleate itself as regards binding to BSA. As shown in Table I, displacement of 16-nitroxyl stearate is slight, if it occurs at all, at fatty acid:BSA ratios below 1.5. The differences shown probably reflect the imprecision of the method when dealing with low unbound fatty acid concentrations. On the other hand, displacement of 16-nitroxyl stearate at ratios of 2.5 and 2.9 is unmistakable.

The displacement by phloretin of oleate from BSA was also shown in Table II by the equilibrium partitioning method of Goodman (2). The limited aqueous solubility of oleate in the absence of BSA was unaffected by phloretin but was increased about 20-fold by addition of BSA (about 750 μg/ml). Since the concentration of unbound fatty acid is fixed by the partition coefficient of the fatty acid between the aqueous and heptane phases, the increase was due to fatty acid bound to BSA. As can be seen, phloretin reduced this binding by about 50%. The effectiveness of phloretin was apparently already maximal at a concentration of 100 μM. Since phloretin reduced the fatty acid:BSA ratio only from 3.77 to about 1.8, its effect

\[
\text{Fatty acid:BSA} = \left(34.4 - 1.64\right)/8.7 = 3.77.
\]

**Table I**

<table>
<thead>
<tr>
<th>Fatty acid:BSA</th>
<th>Control</th>
<th>+Phloretin (200 μM)</th>
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<tr>
<td>molar ratio</td>
<td>μM free 16-nitroxyl stearate</td>
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<td>0.043</td>
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<td>2.9</td>
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</table>

**Fig. 11.** Effect of phloretin on [14C]oleate uptake at different fatty acid (FA):BSA ratios. The BSA concentration was varied from 3.6 to 80 μM while the total fatty acid concentration was maintained at 20 μM. For further experimental details, refer to the legend to Fig. 10. Phloretin was added in ethanol directly to the assay medium.

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**Fig. 9.** Initial rates of [14C]oleate uptake by adipocytes, with and without glucose as a function of unbound fatty acid concentration in the medium. Assays were carried out at 23 °C for 15 s as described under "Experimental Procedures." The concentration of unbound fatty acid was varied by altering the BSA concentration from 3.6 to 80 μM while keeping the fatty acid (FA) constant at 20 μM. The calculation of unbound fatty acid concentration is described under "Experimental Procedures." All cells came from a single batch which was divided in two parts, one of which was maintained and assayed without glucose, whereas the other was maintained and assayed with 2 mM glucose in the medium.

**Fig. 10.** Saturable and nonsaturable components of [14C]oleate uptake. The unbound fatty acid (FA) concentrations were calculated as described under "Experimental Procedures." Cells were held in KRH buffer containing 1% gelatin in order not to disturb the fatty acid:BSA ratio of the assay medium. Otherwise, BSA carried over by the cell suspension lowers the ratio and makes it difficult to obtain values above 2. Gelatin did not bind fatty acid as checked by EPR spectroscopy. Also, similar rates of FA uptake were observed in cells suspended in fatty acid/BSA with or without added gelatin. Uptake was measured at 23 °C for 15 s.

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**Fig. 11.** Uptake of [14C]oleate by adipocytes at different fatty acid (FA):BSA ratios. The BSA concentration was varied from 3.6 to 80 μM while the total fatty acid concentration was maintained at 20 μM. For further experimental details, refer to the legend to Fig. 10. Phloretin was added in ethanol directly to the assay medium.
DISCUSSION

The following developments have helped clarify the nature of fatty acid permeation into adipocytes: 1) use of an effective stop solution to interrupt efflux and influx of fatty acids; 2) separation of permeation from intracellular metabolism and/or binding of fatty acids; 3) estimation of the initial rates of permeation as a function of unbound fatty acid concentration; 4) evaluation of the relationship of the dissociation of fatty acid from albumin to the rate of fatty acid uptake by the cells; and 5) employment of a broad spectrum transport inhibitor to modify fatty acid influx. These points will be discussed below.

Stop Solution—The effectiveness of the stop solution has been described under “Results.” A few points merit further consideration. A stop solution is essential for measurement of fatty acid uptake when a significant fraction of the fatty acid remains in the unesterified form intracellularly. Such would be the case if the incubation time was very short and/or metabolism was limited. The intracellular, unesterified fatty acid can pass very rapidly into wash medium at 0 °C, even in the absence of albumin, leading to an underestimation of uptake and erroneous interpretations. For example, we observed an apparent stimulatory effect of glucose on the initial uptake of fatty acid when buffer alone was used as stop solution. However, this effect was due to loss of unesterified fatty acid from the cells incubated without glucose. The loss was eliminated by inclusion of phloretin in the stop and wash solutions, and the “effect” disappeared.

Permeation versus Metabolism—In previous studies, using a variety of tissues (9–12), metabolism of the fatty acids was always a significant component of the measured uptake rate. It remained uncertain therefore, whether saturation, when observed, reflected behavior of a transport or a metabolic step. We believe that we have separated these processes in the present study. During the first 15 s, the rates of oleate uptake by adipocytes at 23 °C are linear and maximal. They are independent of metabolism, since the rates are the same in the absence or presence of glucose, although fatty acid esterification, which is virtually nonexistent without glucose, is active in its presence (Fig. 9). In the absence of glucose, oleate uptake rates fall off after 15 s. This is due to the beginning of measurable efflux as unesterified fatty acid accumulates. It is prevented when the fatty acid is trapped by esterification, and the initial rate of uptake is then prolonged. The persistence of initial rates in the presence of glucose is consistent with our calculation that dissociation of fatty acid from albumin does not become limiting for uptake. We conclude, therefore, that oleate uptake during the first 15 s represents influx of the fatty acid into the cell.

Binding to the Cell Surface—The possibility that fatty acid uptake reflects binding to the cell surface is unlikely because of the inhibitory effects of phloretin. If uptake were due to binding, phloretin inhibition would be the consequence of occupancy of the fatty acid-binding sites or their allosteric modification by the inhibitor. If this were the case, however, phloretin would be expected to displace fatty acid already bound to the cell; that is, it should stimulate efflux from cells preloaded with unesterified fatty acid. This is contrary to what was observed but is consistent with an action of phloretin at the membrane to prevent influx and efflux of fatty acid.

Comparative Rates of Uptake and/or Transport—It is difficult to compare the rates of oleate uptake we measured to those in other studies because of the different tissues and experimental conditions used. The maximal rate of oleate uptake reported by Samuel et al. (10) in cultured cardiac cells were 0.3 nmol/10⁶ cells/min, as compared to about 2 nmol/10⁶ cells/min in our study of adipocytes. Odom (12) measured linoleate uptake rates in adipocytes as a function of unbound

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**Table II**

<table>
<thead>
<tr>
<th>Addition to aqueous phase</th>
<th>Heptane phase</th>
<th>Aqueous phase</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>998</td>
<td>1.64</td>
</tr>
<tr>
<td>Phloretin (500 μM)</td>
<td>994</td>
<td>1.69</td>
</tr>
<tr>
<td>BSA (8.7 μM)</td>
<td>948</td>
<td>34.4</td>
</tr>
<tr>
<td>BSA + phloretin (100 μM)</td>
<td>978</td>
<td>18.0</td>
</tr>
<tr>
<td>BSA + phloretin (200 μM)</td>
<td>979</td>
<td>17.6</td>
</tr>
<tr>
<td>BSA + phloretin (500 μM)</td>
<td>981</td>
<td>16.5</td>
</tr>
</tbody>
</table>

**Fig. 12.** Inhibition of ¹⁴C-labeled fatty acid uptake by different concentrations of phloretin. Uptake was measured at 23 °C for 15 s at an unbound fatty acid concentration of 0.04 μM (20 μM fatty acid and 40 μM BSA). Phloretin in ethanol or ethanol alone was added directly to the assay medium prior to cell addition. The highest ethanol concentration to which the cells were exposed was 0.5% at 800 μM phloretin. Points are means of duplicates from 2 experiments.

When the displacement of fatty acid from BSA by phloretin is taken into account, it appears that phloretin is inhibitory to uptake of unbound fatty acid at much higher fatty acid:BSA ratios than suggested by Fig. 11. For example, the presence of phloretin at a fatty acid:BSA ratio of 1.8 raises the concentration of unbound fatty acid to the same level as would be obtained with a fatty acid:BSA ratio of 3.77 without phloretin (Fig. 11). The uptake, however, is 20% lower in the presence of phloretin. The degree of inhibition is less than that seen at lower fatty acid:BSA ratios (see also Fig. 12) for reasons which will be discussed.

The inhibition of oleate uptake by increasing concentrations of phloretin is shown in Fig. 12. In this case, the fatty acid:BSA ratio was fixed at 0.5 under which circumstance almost all of the fatty acid is bound to the highest affinity site on the albumin (17, 18). The data presented in Table II suggest that phloretin does not displace fatty acid from this site, even at a concentration of 500 μM. The ratio, 0.5, yields an unbound fatty acid concentration of 0.04 μM, in which circumstance about 90% of the permeation process would occur through the saturable component according to Fig. 10. As will be discussed, it is this presumed transporter element which is sensitive to phloretin inhibition, whereas the diffusion component of permeation may be facilitated, particularly by high concentrations of phloretin. These considerations and the uncertainties of quantitation in the experimental system make the phloretin effect (about 70%) in Fig. 12 not unreasonable in magnitude.

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fatty acid concentration. At the same concentration of unbound fatty acid (0.1 μM), he reports 0.35 nmol/mg/min at 37 °C versus about 0.1 nmol/mg/min in our study at 23 °C. From our experience, we believe that uptake was also limited by transport under the conditions of Odom's study.

The half-maximal rate of oleate transport in our studies at 23 °C was about 270 nmol of carbon atoms/ml of packed cells/min. This compares with a half-maximal rate of about 240 nmol of carbon atoms/ml cell/min for glucose transport without insulin at the same temperature (24). A substantial rate of fatty acid uptake may be necessary to take up fatty acid released from circulating albumin and lipoprotein. Furthermore, a substantial rate for transport of fatty acid from the cell would be expected, since adipose tissue release of fatty acid is the major metabolic source of fuel in fasting.

**Intracellular Binding**—If the unesterified fatty acid accumulating in the adipocyte (without glucose) were in free solution in the cytosol, the calculated concentration would approach 30 times that in the external medium. Pretreatment of the cells with dinitrophenol under conditions which eliminate measurable ATP (25) did not modify the uptake rate (data not shown), but concentration by a Na-coupled cotransport mechanism has not been looked for. If the fatty acid were in free solution, however, its concentration would greatly exceed the aqueous solubility limits of fatty acid in many of our experiments. It seems virtually certain, therefore, that a substantial fraction of the fatty acid is bound to intracellular structures and/or proteins. Fatty acid solubility in triglyceride is not high (26), but many hydrophilic surfaces are present within the cell to which fatty acid could bind. We have found that substantial quantities of fatty acid bind to the microsomal fraction of adipocytes. Judging from the rapid and nearly complete washout of fatty acid under favorable conditions (Figs. 2 and 3), the binding is readily reversible.

**Extracellular Transfer of Fatty Acids**—The rapidity with which intracellular unesterified fatty acid effluxes into the external medium is noteworthy, even at reduced temperature (0 °C) and without added albumin (Figs. 2 and 3). It appears that within 30 s (Fig. 3), equilibrium may be approached between cellular and aqueous fatty acids. It may not be necessary, therefore, to invoke special mechanisms for fatty acid transfer between the intravascular space and the adipocyte, such as lateral diffusion along contiguous cell surfaces (27) or transfer as an albumin complex. We would suggest that the transfer in this case could be regarded as no different in mechanism from that of other small molecules (e.g. glucose) for which effective plasma membrane transport processes are present. The low aqueous solubility of fatty acid is not a barrier to transfer if the rate of efflux into the interstitial water keeps pace with the rate of uptake. In other words, a very low concentration of unbound fatty acid in interstitial water is not incompatible with fatty acid transfer if the turnover rate is high enough.

**Mechanism of Fatty Acid Transfer Across Adipocyte Membranes**—We observed saturation of initial rates of oleate uptake as a function of the unbound fatty acid concentration in the medium. The process exhibited high affinity for oleate (Kₚ = 6 × 10⁻⁴) and could be strongly inhibited by phloretin. It could be argued that fatty acid penetrates the cell by a non-rate-limiting diffusion process and that saturation reflects filling of intracellular binding sites. If this were true, stimulation of esterification by the addition of glucose should unloading these sites and increase uptake rates, particularly as saturation was approached. This is not observed, however (Fig. 9). It also seems unlikely that the inhibitory effect of phloretin on uptake is due to competition for binding sites inside the cell. As argued earlier, phloretin should then enhance oleate efflux from adipocytes preloaded with unesterified fatty acid. This is also contrary to what was observed (Figs. 2 and 3).

Recently, it has been suggested that cellular receptors for albumin are involved in fatty acid uptake by hepatocytes (8). It is probable that albumin does bind to the adipocyte in view of its well known effectiveness in maintaining the physical integrity of the cells in vitro. Our experience to date, however, does not suggest that albumin-cell interaction could account for the saturation of uptake. For example, we observed the same saturation kinetics of oleate uptake when two different methods were employed to generate various concentrations of unbound fatty acid in the medium. In the first, the total content of fatty acid was held constant while the albumin concentration was progressively reduced in order to generate higher fatty acid:BSA ratios and higher unbound fatty acid concentrations. In the second, the albumin was held constant and higher fatty acid:BSA ratios were generated by raising the total fatty acid content. Saturation could not be correlated with albumin concentration in both of these circumstances but could be correlated with the concentration of unbound fatty acid. It is conceivable, however, though unlikely in our view, that albumin-cell interaction is an important step in uptake and that the lowest albumin concentration employed under our conditions (4 μM) was sufficient to support uptake fully.

Phloretin has been shown to inhibit certain membrane transporter proteins (20) and to stimulate diffusion of non-electrolytes through the phospholipid bilayer of erythrocyte membranes (28). In line with this dual effect, phloretin binds to erythrocyte membrane proteins with high affinity and to membrane lipids with low affinity (29). Membrane effects of phloretin obtained with concentrations lower than 100 μM at pH 7.4 are generally inhibitory and reflect interaction with proteins (29). Dual effects of phloretin on the transfer of a series of nonelectrolytes of increasing hydrophobicity were also described. A strong inhibitory effect was observed on the movement of the more hydrophilic compounds like urea and formamide which became less pronounced and eventually turned into an enhancement as the hydrophobic character of the permeant (ethylene glycol, antipyrine) was increased (28). Similarily, phloretin has complex effects on the transfer of thiourea in human red cells (30) which involve a saturable and a nonsaturable (diffusion-like) component. The saturable component was inhibited, while the nonsaturable one seemed to be unaffected. Dual effects of phloretin presumably reflect the transition from a high fractional contribution of pathways involving proteins (inhibited by phloretin) to a relatively high contribution of the lipid diffusion pathways (enhanced by phloretin). It is likely that such a phenomenon is also occurring under our experimental conditions (Fig. 11). At low concentrations of unbound fatty acid, the proportion of ionized fatty acid is relatively great. This form is insoluble in the lipid (25), and fatty acid permeation is presumably primarily protein carrier-mediated. As the concentration of unbound fatty acid is increased, the concentration of relatively lipid-soluble, nonionized fatty acid rises, and diffusion through the lipid pathway becomes significant and may be accelerated by the phloretin. Consistent with the above considerations, phloretin inhibition of oleate uptake was greatest at low fatty acid:BSA ratios and tended to diminish as the fatty acid:BSA ratio (or unbound fatty acid concentration) was increased. Enhancement of the diffusion component may account for the apparent diminution of inhibition when the phloretin concentration was increased at a fixed fatty acid:BSA ratio (Fig. 12).

In conclusion, our results indicate that a saturable carrier mechanism facilitates the entry of fatty acid into adipocytes. Preliminary results obtained with membrane active agents
like pronase and 4,4'-diisothiocyanostilbene-2,2'-disulfonate further support this conclusion. The possibility that a protein analogous to the anion-binding transporter described for the erythrocyte (31) is involved in fatty acid transfer is an attractive hypothesis. Phloretin and fatty acids (decanoate and octanoate) have been shown to inhibit anion influx into erythrocytes (31). This hypothesis is now being investigated in further detail. At physiological concentrations of unbound fatty acids, this mechanism would account for virtually all fatty acid permeation.

Note Added in Proof—We are grateful to the editors for calling our attention to a paper by Nunn, W. D., Simons, R. W., Egan, P. A., and Maloy, S. R. (1979) J. Biol. Chem. 254, 9130-9134. The authors have substantial evidence to 'suggest that the fadL gene governs a transport component(s) which is required for optimal transport of fatty acids with chain lengths greater than 9 carbon atoms.'

Acknowledgments—We wish to thank Dr. David M. Regen for his critical advice and for reviewing the manuscript, Drs. Tetsuro Kono, Albert Beth, and K. Balasubramanian for many helpful suggestions, and Maylene Long for her help in preparing the manuscript.

REFERENCES