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Hepatocellular influx of [^{14}C]oleate reflects membrane transport rather than intracellular metabolism or binding

(free fatty acids/hepatocytes/hepatic uptake/carrier-mediated transport)

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ABSTRACT **When [^{14}C]oleate bound to bovine serum albumin was incubated at 37°C for 7 min with isolated rat hepatocytes in the absence of glucose, the cumulative oleate uptake curve had two components: a rapid, linear segment over the first 30 sec, followed by a slower, curvilinear component. At 173 μM [^{14}C]oleate/albumin (1:1, mol/mol), the initial component had a slope (V_0) of 118 ± 18 pmol per min per 5 × 10^4 hepatocytes (mean ± SD). During this initial 30 sec, virtually no oleate was oxidized, and <11% was esterified. By 5 min, 79% was esterified; oxidation never exceeded 4%. Addition of 2 mM glucose significantly increased oleate esterification and thereby available oleate binding sites on cytosolic fatty acid binding protein but had no influence on V_0. Pretreatment with trypsin reduced V_0 by 49 ± 15%. These data indicate that the initial component of the oleate uptake curve reflects predominantly influx, whereas the subsequent component reflects a balance between influx, efflux, and intracellular metabolism. Influx is independent of intracellular binding, oxidation, and esterification and may reflect a membrane-associated carrier-mediated process.**

The hepatocellular uptake process for long-chain fatty acids is still incompletely understood. Although cellular permeation of fatty acids is widely considered to occur by diffusion (1–3), some studies report kinetic features such as saturation suggestive of a carrier-mediated process (4–10). Other investigators (11, 12) have argued that this apparent saturation of uptake, as well as the effects on uptake produced by sex hormones (13), fasting (14), and clofibrate (15), reflected saturation of an intracellular metabolic step rather than of membrane transport. Therefore, the aim of the present study was to examine the influence of intracellular metabolism and binding on uptake of a representative long-chain fatty acid, [^{14}C]oleate, by isolated rat hepatocytes.

MATERIALS AND METHODS

Preparation of Hepatocytes. Hepatocytes of nonfasted male Sprague–Dawley rats (290–300 g) were prepared by the procedure of Berry and Friend (16) using as the perfusion medium a calcium-free buffer containing 140 mM NaCl, 5 mM KCl, 0.8 mM $MgSO_4 \cdot 7\ H_2O$, 0.4 mM KH_2PO_4, 5 mM glucose, 5 mM Hepes, 0.3 mM $Na_2HPO_4 \cdot 12\ H_2O$, oxygenated for 5 min with 100% O_2 at pH 7.4 and 37°C. Viability was routinely tested. Cells were considered suitable when more than 90% excluded trypan blue before and after each experiment. Additional criteria for viability were an intracellular K^+ concentration >85 mM, less than 15% loss of lactate dehydrogenase, and less than 20% stimulation of respiration by succinate. The purified homogeneous cell suspension of hepatic parenchymal cells was washed and resuspended three times in 0.15 M NaCl/0.02 M sodium phosphate (pH 7.4, PBS), diluted to a concentration of 2 × 10^6 cells per ml and kept on ice until use. The average protein content (17) of the isolated hepatocytes was 2.19 ± 0.21 mg of protein per 10^6 cells.

Assay of Fatty Acid Uptake. [1-^{14}C]Oleate (900 mCi/mmol; 1 Ci = 37 GBq; 99% radiochemically pure; New England Nuclear) was mixed with measured quantities of nonradioactive oleic acid (Sigma) in chloroform, dried under a N_2 gas stream, and dissolved in 0.2 ml of 0.1 N NaOH at 37°C. Defatted (6) bovine serum albumin (BSA) (fraction V, Sigma) dissolved in PBS was added to the oleate/NaOH solution to obtain the desired oleate/albumin molar ratio, the pH was adjusted to 7.4, and the oleate/BSA solution was diluted to its final working concentration in PBS. After bringing both cells and substrates to the appropriate temperature, the [^{14}C]oleate solutions were incubated with continuous mixing with 30- to 250-μl aliquots of the hepatocyte suspension (2 × 10^6 cells per ml) in polypropylene tubes in a final volume of 1 ml PBS, pH 7.4. These specific tubes were used since incubations with the various molar ratios of [^{14}C]-oleate/albumin in solution in the absence of cells showed no change of [^{14}C]oleate concentrations with time indicating that, under the conditions employed, oleate does not dissolve into polypropylene. At various time intervals oleate uptake was stopped by pipetting 100 μl of the incubation mixture into 1 ml of PBS containing 200 μM phloretin and 0.1% BSA (stop/chase solution) at 4°C. The sample was immediately pipetted onto the center of a Whatman GF/C filter (24 mm) at a rate equal to the rate of filtration under 50 mm of Hg vacuum pressure using a filtration apparatus (model 7H, Hoefer, San Francisco) (18). The cells were washed with 5 ml of the stop/chase solution and, thereafter, with 20 ml of PBS. Washing with more of the stop solution did not change the radioactivity remaining on the filter. The filter was placed in scintillation vials and left overnight in 1 ml of Protosol (New England Nuclear). Subsequently, 10 ml of Econofluor (New England Nuclear) was added, and the radioactivity was determined in a 1217 Rackbeta liquid scintillation counter (LKB-Wallac, Turku, Finland) at a counting efficiency of 92%. Adsorption of radiolabeled oleate to the filters was routinely determined by replacing the cell suspension in the incubation with PBS. It constituted less than 4% of the incubated radioactivity and was dependent on the BSA concentration in the buffer. Even in samples with high BSA concentrations and low rates of cellular oleate uptake, blanks never exceeded 32% of the uptake value and were usually substantially smaller. The radiolabeled oleate adsorbed to the filters without cells was very reproducible for any given experimental circumstance and was routinely determined and subtracted from values measured with cells.

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Abbreviation: BSA, bovine serum albumin.

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Separation of Cellular Lipids. The incorporation of [^{14}C]oleate into cellular lipids was determined after incubation of 173 μM [^{14}C]oleate/albumin (1:1, mol/mol) with 5 × 10^5 hepatocytes in 2 ml of PBS at 37°C for 0.5, 1, 2.5, or 5 min. Incubations were conducted both in the presence and absence of 2 mM glucose. For filtration, filters resistant to organic solvents (SCWP, Millipore) were used. Filters plus cells were extracted three times in 4 ml of chloroform/methanol (2:1, vol/vol). The extracts were pooled and 0.2 vol of water was added to separate out an aqueous phase. The chloroform phase was evaporated under a stream of nitrogen, then redissolved in 200 μl of chloroform/methanol (2:1, vol/vol), and applied to silica gel plates (DC - Fertigplatte Kieselgel 60 F_{254}, Merck, Darmstadt, F.R.G.). The developing solvent was petroleum ether/diethyl ether/acetic acid (80:20:1, vol/vol). The bands were visualized by 254-nm UV-light and identified by comparison to standards that were run simultaneously. Radioactivity in the bands was counted in 10 ml of Aquasol (New England Nuclear). For further separation of free oleic acid from the glyceride fractions, corresponding lipid zones were eluted from the silica gel by three extractions with a methanol/chloroform/heptane mixture (1.41:1.25:1, vol/vol) and further analyzed by the procedure of Belfrage and Vaughan (19) (recovery >98%).

Assay of [^{14}C]Oleate Oxidation to CO_2. For these studies incubations were carried out as described above, in rubber capped flasks with a separated center dish containing 0.3 ml of monoethanolamine (10). At various times 0.2 ml of 2.5 M H_2SO_4 was injected into the reaction medium through the rubber cap to stop the reaction and to liberate CO_2. After 1 hr at 37°C, 0.2 ml of the monoethanolamine containing the trapped $^{14}CO_2$ was transferred to scintillation vials for counting with 10 ml of Aquasol. In control experiments radioactive bicarbonate was completely recovered in monoethanolamine under these conditions.

Trypsin Pretreatment of Hepatocytes. The effect of trypsin pretreatment of hepatocytes on [^{14}C]oleate influx was determined by preincubation of 2 ml of the hepatocyte suspension (2 × 10^6 cells per ml) with 1 ml of trypsin solution (0.6 mg/ml) (Sigma) in 10 mM Tris·HCl, pH 8.0, for 15 min at 37°C. The cells were then washed three times with PBS, centrifuged, and resuspended to 2 × 10^6 cells per ml. Controls were incubated with 10 mM Tris·HCl, pH 8, without trypsin and, thereafter, washed, centrifuged, and resuspended as above. The viability of the cells after preincubation was >90%. The cell suspension (250 μl) was then incubated with 173 μM [^{14}C]oleate/BSA (1:1, mol/mol) in 1 ml of PBS at 37°C.

RESULTS

Validation of Methods. To measure uptake accurately and distinguish internalized ligand from that merely membrane bound, it is essential to employ a stop solution that blocks influx and efflux after certain periods of incubation and removes membrane bound but not transported ligand. Phloretin efficiently inhibits cellular transport systems (20–22) and has been reported to block both influx and efflux of fatty acids by adipocytes (7). To examine its effect in hepatocytes, cell suspensions were incubated at 37°C with various concentrations of [^{14}C]oleate, alone or in the presence of specific concentrations of BSA, for 15 or 30 sec, with or without 2 mM glucose. At zero time, 10–20 vol of a specified stop solution at either 37°C or 4°C was added, and radioactivity remaining with the cells was determined at intervals up to 5 min. In representative studies such as the one shown in Fig. 1*A*, use of PBS at 4°C as stop solution was followed by a gradual loss of cellular radioactivity; addition of 200 μM phloretin to the PBS essentially abolished these losses. Addition of various concentrations of BSA (0.1–1%; 15–150 μM) to the PBS stop solution resulted in a virtually instantaneous loss of approximately 55% of cell-associated radioactivity, followed by a slow but progressive loss of an additional 15–35% over the subsequent 5 min. *In toto* up to 80% of initial cell-associated radioactivity was released into buffer containing 1% BSA without phloretin. These results suggest that the bulk of cell-associated [^{14}C]oleate after a 30-sec incubation is still unesterified, which was confirmed subsequently by direct measurement (*vide infra*). That the initial instantaneous loss observed in the presence of albumin represents [^{14}C]oleate effectively chased off membrane binding sites while the later, slower losses represent efflux of internalized [^{14}C]oleate is supported by the following observations. (*i*) The instantaneous loss of cell-associated radioactivity was essentially identical for all concentrations of BSA from 0.1–1%, despite wide ranges in the final oleate/albumin molar ratios and consequent unbound oleate concentrations. (*ii*) Incubation of hepatocytes with [^{14}C]oleate at 4°C for 30 sec, conditions under which little ligand would be expected to be internalized, resulted in baseline levels of cell-associated radioactivity averaging 56% of those observed at 37°C. Subsequent addition of 0.1 or 1% BSA stop solutions at 4°C to such cells resulted in the instantaneous loss of ≤90% of this cell-associated radioactivity. Moreover, these losses were not prevented by addition of 200 μM phloretin to the stop solutions (Fig. 1*B*). (*iii*) Following preloading of cells with [^{14}C]oleate at 37°C, addition of a stop solution containing 0.1% BSA and 200 μM phloretin led to instantaneous losses similar to those observed with albumin alone, but no further depletion of cell-associated radioactivity (Fig. 1*A*). When 200 μM phloretin was added to stop solutions containing higher BSA concentrations (0.2%–1.0%), instantaneous losses identical to those seen with 0.1% BSA were followed by progressive efflux of [^{14}C]oleate at rates that increased with the albumin concentration employed and became appreciable at concentrations ≥0.6%. Thus, as observed in adipocytes (7), increasing concentrations of BSA can overcome the effects of phloretin on fatty acid efflux. These studies suggest that the combination of 0.1% BSA and 200 μM phloretin at 4°C provides an effective stop/chase solution for removal of fatty acid molecules bound to the cell membrane, while preventing efflux of internalized ligand.

Uptake of [^{14}C]Oleate by Isolated Hepatocytes. Incubation of 173 μM [^{14}C]oleate/BSA (1:1, mol/mol) with 5 × 10^5 hepatocytes at 37°C in the absence of glucose revealed that the slope of the cumulative uptake curve was maximal and linear over the first 30 sec (Fig. 2). It gradually decreased over the next 30 sec and then remained constant between 1 and 7 min, during which time the cells continued to accumulate ligand. Cumulative uptake curves measured in the presence and absence of glucose were virtually identical (Fig. 2). The initial [^{14}C]oleate uptake rates (V_o) were taken to be the slopes of the cumulative uptake curves over the initial 30-sec period and corresponded to 118 ± 18 pmol per min per 5 × 10^4 hepatocytes. While the initial linear uptake component predominantly reflects influx, after 30 sec the cumulative uptake curve represents net accumulation by the cells including influx, efflux, and intracellular metabolism. This was demonstrated in part by the increasing proportions of [^{14}C]oleate present in esterified form or oxidized to $^{14}CO_2$ with increasing time of incubation. In a representative study, 89.4 ± 5.1% of the [^{14}C]oleate taken up by the cells at 30 sec was recovered in unesterified form. After a 1-min incubation 77.2 ± 3.3% of the oleate was still unesterified, while after 2.5 min 37.9 ± 3.7% and after 5 min 21.2 ± 3.5% was nonesterified. Similarly oxidation of [^{14}C]oleate to $^{14}CO_2$ was not measurable within the first 2 min of incubation. However, after 2.5 min 0.68 ± 0.11 pmol of [^{14}C]oleate per 5 × 10^4 hepatocytes was recovered as $^{14}CO_2$, and after 30 min, 4.4 ± 0.9 pmol of [^{14}C]oleate per 5 × 10^4 cells were recovered. The

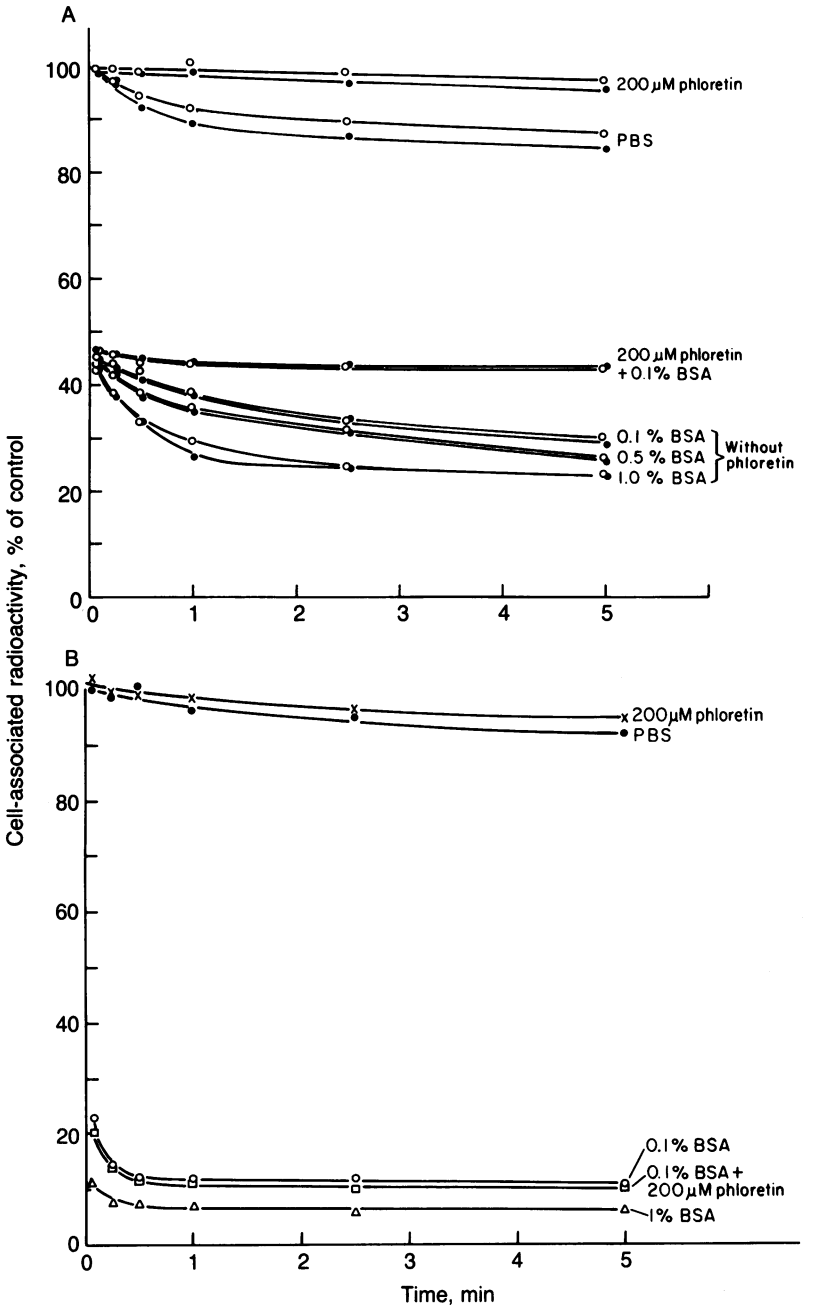

FIG. 1. (*A*) Effect of various potential "stop solutions" on cell-associated [^{14}C]oleate. Hepatocyte suspension (30 μl, 4 × 10^6 cells per ml), isolated and assayed in the presence (●) and absence (○) of glucose, were incubated for 15 sec at 37°C with 20 μl of 50 μM [^{14}C]oleate and 100 μM BSA. At zero time, 1 ml of stop solution at 4°C was added. At the times shown on the abscissa, 5 ml of 200 μM phloretin (4°C) was added, and the reaction mixture was immediately filtered, and the cell-associated radioactivity was determined. Controls corresponding to 100% radioactivity were cells to which 5 ml of cold phloretin solution (4°C) was added immediately at the end of the 15-sec incubation period. Values illustrated are means of three observations. (*B*) Loss of cell-associated radioactivity from rat hepatocytes preincubated with [^{14}C]oleate at 4°C. Hepatocyte suspension (30 μl) was incubated for 15 sec at 4°C with 50 μM [^{14}C]oleate and 100 μM BSA. At zero time, 1 ml of stop solution at 4°C was added. At the times shown on the abscissa, 5 ml of 200 μM phloretin (4°C) was added, and the reaction mixture was immediately filtered, and the cell-associated radioactivity was determined. Controls corresponding to 100% radioactivity were cells to which 5 ml of cold phloretin solution (4°C) was added immediately at the end of the 15-sec incubation period. Values illustrated are means of three observations.

latter result corresponded to less than 4% of the oleate taken up. Finally, the initial uptake velocity of [^{14}C]oleate was compared in hepatocytes incubated with or without glucose. V_o in the presence of glucose average 100 ± 3.5% of that in the absence of glucose (Fig. 2), although the proportion of sequestered fatty acids present in unesterified form at 30 sec in the presence of glucose (76 ± 12%) was significantly less ($P < 0.01$) than that observed in its absence (89 ± 7%). These studies confirm that the initial slope of the cumulative uptake curves over the first 30-sec incubation predominantly reflect influx and not metabolism.

Within the hepatocyte and enterocyte, newly sequestered nonesterified fatty acids are found principally bound to a low molecular weight (≈12–14 kDa), cytosolic, fatty acid-binding protein, which does not bind fatty acid esters (23–25). The observed differences in unesterified fatty acids in cells incubated with and without glucose imply a difference in unoccupied binding sites on the cytosolic, fatty acid-binding protein, which had no effect on V_o. In further studies, V_o was determined during preloading of hepatocytes with 173 μM [^{3}H]oleate/albumin (1:1, mol/mol) for 30 sec in the absence of glucose, after which [^{14}C]oleate/albumin (1:1, mol/mol) was added to the incubation. V_o of the [^{14}C]oleate into the preloaded cells over the subsequent 30 sec was identical to that of [^{3}H]oleate observed initially. These studies suggest that V_o, as measured over the initial 30 sec, is not determined to a significant degree by availability of intracellular binding sites.

Effect of Trypsin Pretreatment of Hepatocytes on Uptake of [^{14}C]Oleate. Pretreatment of hepatocytes with trypsin yielded a 49 ± 15% reduction in V_o, from 118 ± 18 to 60 ± 18 pmol per min per 5 × 10^4 hepatocytes ($P < 0.01$), indicating that at least a portion of hepatocellular uptake of fatty acid is protein mediated.

DISCUSSION

In the present studies experimental conditions were established by which hepatocellular uptake of a representative long-chain fatty acid, [^{14}C]oleate, could reliably be deter-

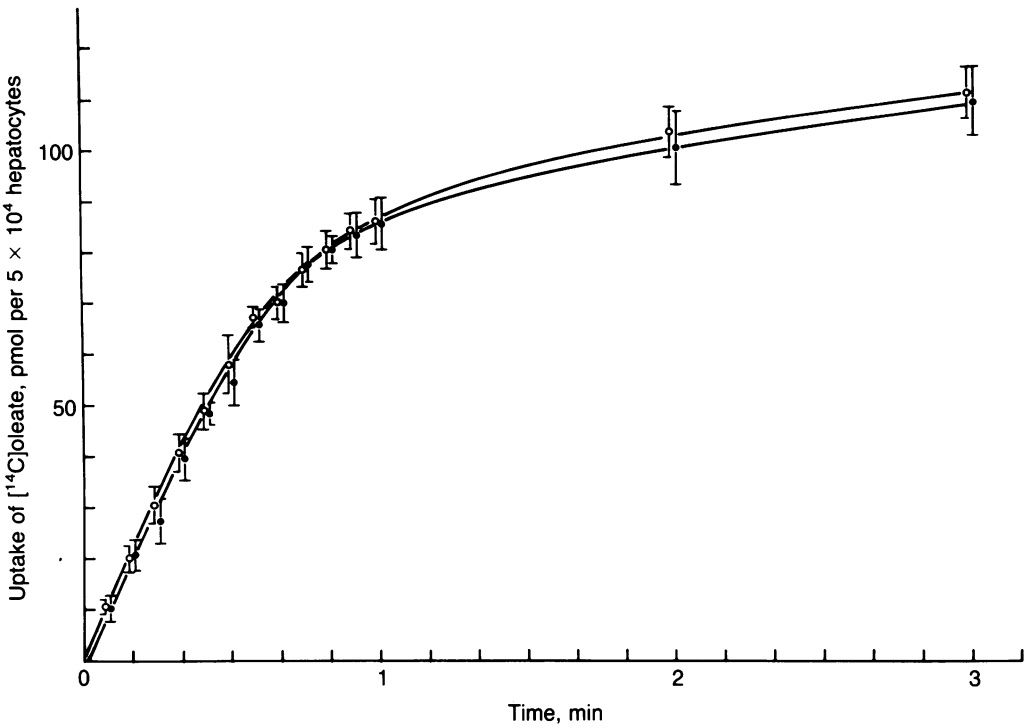

FIG. 2. Time course of [^{14}C]oleate uptake in the absence (○) and presence (●) of glucose. Values are means ± SD of three observations.

mined by direct measurement of cell-associated radioactivity. The use of 200 μM phloretin/0.1% BSA as stop/chase solution provides that (*i*) cellular efflux of fatty acids taken up by the hepatocytes was inhibited during the experimental procedure, and (*ii*) only internalized, not membrane bound, ligand was measured. In the system of isolated hepatocytes employed, the cumulative fatty acid uptake curve is composed of two components: a rapid, initial uptake phase representing cellular influx and a later, slowly increasing accumulation period representing the net result of influx, efflux, and intracellular metabolism. The linearity of the initial influx component over the first 30-sec incubation period suggested that metabolism and efflux were of minor significance during this period. This was supported by the fact that 89% of [^{14}C]oleate recovered in the cells during this period remained in the unesterified form and oxidation to $^{14}CO_2$ was virtually undetectable. In contrast, during the second component of the cumulative uptake curve, the esterification process gradually increased such that, after 5 min, 79% of the fatty acids were esterified. Oxidation also increased, although quantitatively to a much lesser extent. These results indicate that hepatocellular influx of long-chain fatty acids is independent of cellular metabolism. That fatty acid uptake velocity was identical in the presence and absence of glucose, which significantly alters the extent of esterification and thereby, the availability of unoccupied binding sites on cytosolic fatty acid binding protein, also indicates that uptake is independent of such cytosolic binding, as confirmed by dual isotope studies.

Whether the initial uptake rate of fatty acids reflects a diffusional process or involves a membrane-associated carrier mechanism is not established by the experiments just cited. Since most membrane-carrier processes involve surface membrane proteins, the effect of trypsin pretreatment of the hepatocytes on cellular influx of [^{14}C]oleate was determined. The initial uptake rate of [^{14}C]oleate was markedly reduced in trypsin-pretreated hepatocytes under conditions that did not alter their viability. The suggestion that a protein might be involved in the hepatocellular uptake of long-chain fatty acids is consistent with the previous identification of a fatty acid-binding membrane protein in rat liver plasma membranes (6). However, the physiologic significance of this membrane protein in the hepatocellular uptake of long-chain fatty acids remains to be established.

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