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STUDIES OF OLEATE BINDING TO RAT LIVER PLASMA MEMBRANES

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The binding of [^{14}C]oleate to rat liver plasma membranes was examined under various conditions in vitro. In protein free incubation mixtures binding was saturable, inhibited by excess cold oleate, virtually abolished by heat denaturation of the membranes, reversible by post-incubation with cold oleate or albumin, and pH and temperature dependent. When [^{14}C]oleate was incubated in the presence of albumin or β-lactoglobulin, the amount of membrane bound oleate was a function of the calculated free oleate concentration in the incubation mixture. Trypsin significantly inhibited binding. Further analysis suggests that membranes contain 10^{15} high affinity (K_{av} = 2 x $10^{8}M^{-1}$) oleate binding sites/mg protein.

Several classes of low molecular weight compounds - including bile acids, non-bile acid cholephilic anions such as sulfobromophthalein (BSP) and bilirubin, and fatty acids - are efficiently extracted by the liver despite very tight binding to albumin within the circulation (1). The kinetics of hepatocellular uptake of bile acids and of BSP and bilirubin are suggestive of carrier mediated transport (1), and putative membrane receptor proteins have been identified for bile acids (2) and isolated for BSP and bilirubin (3-5). Less information is available about the hepatocytic uptake of fatty acids. Since binding to plasma membranes is the initial step in a carrier mediated uptake process, the aim of the present study was to characterize the binding of a representative fatty acid, oleate, to rat liver sinusoidal plasma membranes.

ABBREVIATIONS: LPM = rat liver plasma membranes.

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MATERIALS AND METHODS

Preparation of Rat Liver Plasma Membranes (LPM). LPM enriched in sinusoidal components were prepared from the livers of Sprague-Dawley rats by differential centrifugation (6,7), characterized by electron microscopy (8) and analysis of appropriate enzymatic markers (7), and stored at -80°C in aliquots containing 5 mg membrane protein (9) until use. Membrane preparations were free of albumin by double immunodiffusion in agar (10) employing a rabbit anti-rat serum albumin (Cappel Laboratories, Downington, PA).

Incubation Procedures. [^{14}C]oleate in ethanol (900 mCi/mmol; 99% radiochemically pure; New England Nuclear, Boston, MA) was mixed with known quantities of unlabeled oleate (Sigma, St. Louis, MO) in appropriate volumes of 1 mM $NaHCO_3$ buffer, pH 7.6. To minimize non-specific precipitation in the binding experiments, these [^{14}C]oleate suspensions (11) were pre-incubated by rotation in polypropylene tubes for 30 min at room temperature (22°C), and centrifuged for 15 minutes at 15,000 g. The supernatant, in which the final oleate content was estimated by isotopic dilution, was employed in subsequent LPM binding studies. Incubations were conducted with [^{14}C]oleate alone, or with [^{14}C]oleate bound either to rat albumin or β-lactoglobulin (Sigma). In the latter instances, the proteins were first defatted (12), before addition to the [^{14}C]oleate preparations in specific amounts, such that the oleate: protein molar ratio varied from 0.25:1 to 10:1.

In typical incubations, aliquots of LPM (5 mg protein) were thawed, centrifuged for 15 min at 15,000 g, and resuspended in the appropriate [^{14}C]oleate preparation, which contained 0.02-2,500 nmoles oleate with or without albumin or β-lactoglobulin. The incubation mixtures were usually made up to 3 ml with 1 mM $NaHCO_3$ buffer, pH 7.6, but incubation volumes of 1 or 4 ml were occasionally employed. Incubations were carried out for 30 min at room temperature, after preliminary studies had established that equilibration was complete within this period.

Binding Assay. Binding was initially assessed from the distribution of radioactivity between membrane pellet and supernatant following both an initial centrifugation (15,000 g, 15 min) and after 4 cycles of resuspending the pellet in bicarbonate buffer (1 mM, pH 7.6) and recentrifugation. Subsequently, after establishing that further washing cycles removed less than 10% of the remaining radioactivity per cycle, the radioactivity in the pellet after the fourth recentrifugation was used as an estimate of binding (13). Radioactivity was determined in Aquasol (New England Nuclear) by liquid scintillation counting (Mark III, Traycor Analytic, Elk Grove Village, IL). Efficiency was 92%, as determined by a channels ratio method with a ^{133}Ba external standard.

For incubations incorporating albumin or β-lactoglobulin, the equilibrium concentration of free oleate in the incubation mixtures was estimated from the published stepwise equilibrium binding constants of the two proteins (14,15), employing a TI-58C programable calculator (Texas Instruments, Dallas, TX). For these computations, the total oleate concentration in solution in equilibrium with the protein in question was estimated from the total oleate added to the system minus the amount recovered bound to the membranes.

Effects of Enzymatic Digestion. Aliquots of LPM (5 mg protein) were pre-incubated for 30 min at 37° with one of the following enzymes: 110 U BAEE trypsin (Sigma) with and without an excess of soybean trypsin inhibitor (Calbiochem-Behring Corp., La Jolla, CA); 2 U phospholipase C (Type I from Cl. perfringens, Sigma); 2 U neuraminidase (Cl. perfringens, Sigma) in 10 mM monosodium/disodium phosphate buffer, pH 7.6; or 8.1 U phospholipase A_2 (Sigma) in 10 mM tris-maleate buffer, pH 7.4. To the digested membrane suspensions (0.75 ml volume), 0.25 ml of a solution of 21.2 μM [^{14}C]oleate: 85 μM

rat albumin was added, with incubation for 30 min at room temperature. Binding of [^{14}C]oleate to control and treated membranes was determined as above.

RESULTS

Characterization of LPM Fractions. The isolated LPM fractions were similar to those reported in previous studies (7), and were enriched 25-fold in 5'-nucleotidase and 12-fold in glucagon stimulated adenylate cyclase compared to the initial homogenate. Contamination by other organelles was negligible, as assessed by both marker enzymes and electron microscopy.

Binding of [^{14}C]Oleate to LPM. The absolute quantities of [^{14}C]oleate which bound to LPM varied somewhat with different membrane preparations. However, the effects on binding of specific changes in conditions (temperature, pH, cold oleate, enzymatic digestion) were internally consistent with each batch of membranes employed. In a typical experiment (Figure 1), when 440 nmoles [^{14}C]oleate, without either albumin or β-lactoglobulin, were incubated with LPM (5 mg protein), more than 90% of the incubated oleate was

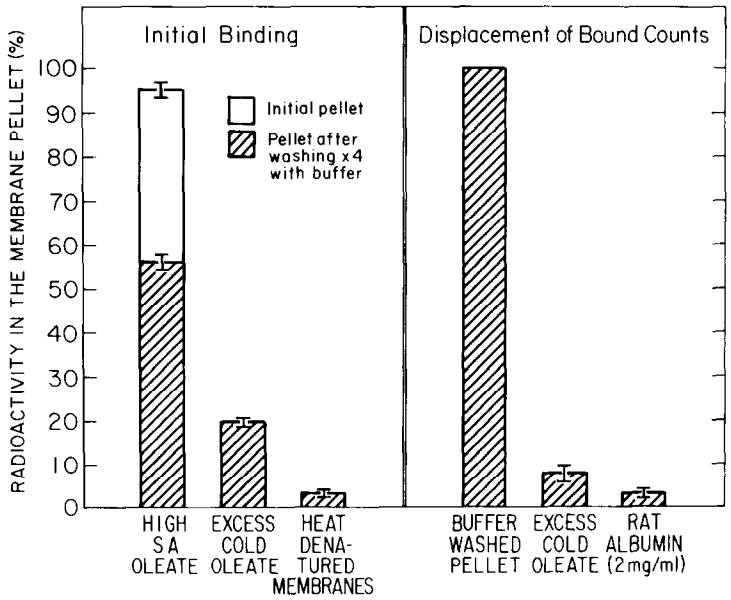

Figure 1. Replicate studies of [^{14}C]oleate binding to rat LPM from a protein-free incubation medium. Left hand panel illustrates retention of membrane bound counts despite repeated buffer washes, as well as the inhibitory effects on [^{14}C]oleate binding of excess cold oleate and heat denaturation of the membranes. Right hand panel illustrates displacement of bound counts from the buffer-washed membranes by post-incubation with either cold oleate or rat albumin.

recovered in the initial membrane pellet, and more than 50% remained after 4 cycles of washing and recentrifugation. All subsequent binding data are in terms of radioactivity in the washed, recentrifuged pellets.

Binding of [^{14}C]oleate was reduced more than 60% by inclusion of a 10-fold excess of cold oleate in the incubation, and was virtually abolished by heating the membranes to 100°C for 5 minutes before incubation. More than 90% of membrane bound oleate was displaced by post-incubation with either a 10-fold excess of cold oleate or with rat albumin (2 mg/ml) (Figure 1).

In the absence of albumin and β-lactoglobulin, oleate binding to LPM was saturable, and had temperature and pH optima at 37°C and pH 7.2, respectively. At 22°C, the maximal binding capacity, representing both specifically and non-specifically bound oleate, was 42 nmol/mg membrane protein.

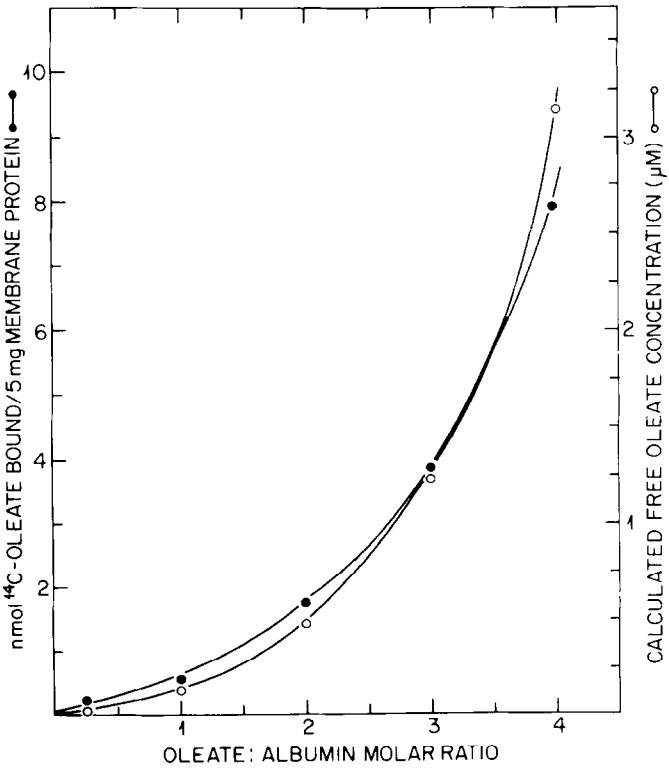

Figure 2. Effect of albumin: oleate molar ratio on [^{14}C]oleate binding to LPM. All incubations contained 21.25 μM rat albumin; oleate concentration varied from 5.3 to 85 μM. The binding curve of oleate to LPM observed at different oleate:albumin molar ratios (●) was superimposable on the calculated free oleate concentrations in the incubation mixtures (○).

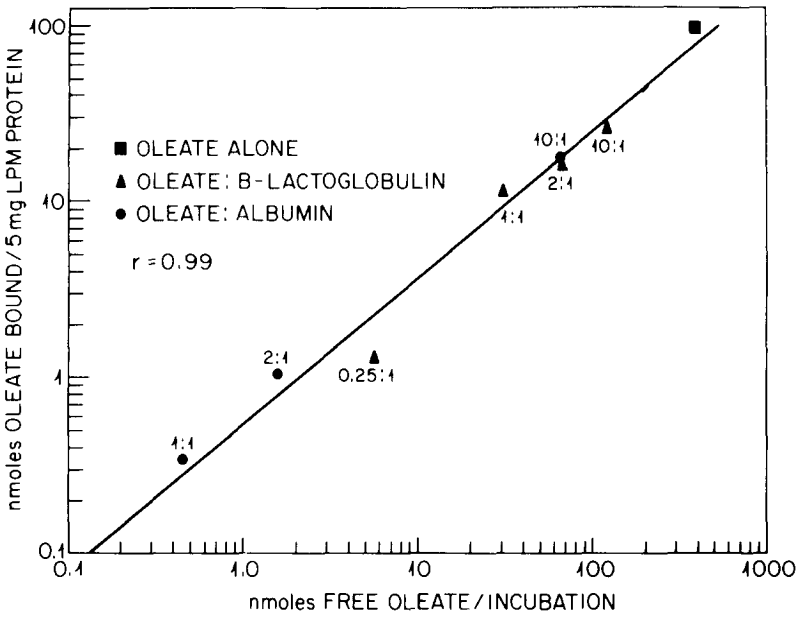

Figure 3. Binding of [^{14}C]oleate to LPM as a function of the unbound oleate concentration. [^{14}C]Oleate was incubated with LPM alone (■) or in the form of complexes with albumin (●) or β-lactoglobulin (▲). Complexes had oleate: protein molar ratios ranging from 0.25 to 10.0. The amount of oleate bound to LPM varied as a function of the calculated free oleate concentration in the incubation mixture, and was not dependent on either the total oleate concentration or the nature of the binding protein.

Influence of Albumin and β-Lactoglobulin. Incubation of 15.8 to 252 nmoles of [^{14}C]oleate with LPM in the presence of 63 nmoles of albumin (molar ratio = 0.25-4) produced an exponential binding curve; its shape was superimposable on the curve obtained with equivalent quantities of oleate without albumin. Both in these studies (Figure 2) and in additional incubations containing oleate:albumin or oleate:β-lactoglobulin at molar ratios ranging from 0.25:1 to 10:1 (Figure 3), membrane bound [^{14}C]oleate was a function of the calculated amount of free oleate in the incubation mixtures. Analysis of the data in Figure 3 by a modification of the Langmuir binding isotherm (16) indicates that each mg of LPM membrane protein contains 1 x 10^{15} high affinity oleate binding sites having a K_{av} of 2.0 x 10^{8} M^{-1}.

Effects of Enzyme Digestion. Oleate binding to LMP was reduced to 51±2% and 49±3% of control, respectively, by trypsin and phospholipase A_2 ($p<0.005$), but was unchanged by phospholipase C or neuraminidase. The effect of trypsin on binding was completely inhibited by soybean trypsin inhibitor.

DISCUSSION

Although the uptake of fatty acids into cells, including hepatocytes, is often described as a passive, diffusional process (17,18), recent studies have suggested more complex mechanisms (19-23). The present studies were undertaken to investigate whether the binding of fatty acids to liver plasma membranes paralleled that already described for bile acids (24) and for BSP and bilirubin (5,7), the other two classes of albumin bound organic anions effectively sequestered by the liver. However, interpretation of the data is complicated by the low solubility of fatty acids in aqueous media. Our albumin-free incubation studies incorporated oleate concentrations of 0.01-800 μM, with apparent saturation being observed at total oleate concentrations in excess of 250 μM. As long chain fatty acids self-associate at concentrations exceeding 1-10 μM, the actual concentrations of oleate monomer in many of the incubation mixtures cannot be precisely determined. Moreover, attempts to distinguish specific from non-specific binding by incubation of tracer with the conventional 100-fold excess of cold ligand were not feasible because of the insolubility of the required amounts of oleate. Finally, it is now recognized that the interaction between organic anions with prominent hydrophobic regions and synthetic lipid bilayer liposomes may show many features of "specific" binding (25,26). Hence, while the observation that oleate binding to liver plasma membranes is saturable, inhibitable, reversible, and temperature and pH dependent suggests specific binding, it does not constitute proof. Computer analysis of our oleate binding data suggests non-specific binding as well as multiple classes of specific binding sites, but good convergence of the data with models incorporating up to three classes of specific sites was not achieved. Additional binding data were obtained in the presence of albumin and β-lactoglobulin, in order to minimize the binding of oleate both to specific sites with low affinity and to various non-specific membrane components (27,28). When analyzed according to Langmuir (16), these studies suggest that about 4% of the maximal oleate binding capacity of LPM was related to a high affinity binding site having an association constant an order of

magnitude higher than the highest affinity site for oleate on the albumin molecule (14). These results correspond to the fraction of membrane-bound oleate not displaceable by post-incubation with albumin (Figure 1), and are similar to those for the membrane binding of BSP (7). Enzyme digestion studies also suggested that a portion of oleate binding to LPM might be due to a specific membrane protein.

While not conclusive, the data presented above were sufficiently suggestive of specific binding to warrant a search for an oleate binding membrane protein. Since preliminary studies suggest that rat liver plasma membranes do contain such a protein (13), our current working hypothesis is that each of the three major classes of albumin bound organic anions sequestered by the liver is taken up via its own discrete, membrane carrier system.

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