Fatty Acid Transfer between Multilamellar Liposomes and Fatty Acid-binding Proteins*

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A simple experimental system was developed for studying the movement of long-chain fatty acids between multilamellar liposomes and soluble proteins capable of binding fatty acids. Oleic acid was incorporated into multilamellar liposomes containing cholesterol and egg yolk lecithin and incubated with albumin or hepatic fatty acid-binding protein. It was found that the fatty acid transferred from the liposomes to either protein rapidly and selectively under conditions where phospholipid and cholesterol transfer did not occur. More than 50% of the fatty acid contained within liposomes could become protein bound, suggesting that the fatty acid moved readily between and across phospholipid bilayers. Transfer was reduced at low pH, and this reduction appeared to result from decreased dissociation of the protonated fatty acid from the bilayer. Liposomes made with dimyristoyl or dipalmitoyl lecithin and containing 1 mol per cent palmitic acid were used to show the effect of temperature on fatty acid transfer. Transfer to either protein did not occur at temperatures where the liposomes were in a gel state but occurred rapidly at temperatures at or above the transition temperatures of the phospholipid used.

Although the interaction between long-chain fatty acids and proteins, particularly albumin, has been characterized (1, 2), less information is available on the nature of the interaction between protein-bound fatty acid and membranes. A biologically relevant aspect of such interactions is the mechanism by which fatty acids cross cell membranes. Prior to entering a cell, fatty acids are usually either complexed to albumin or are generated by lipolytic events occurring on the extracellular side of the cell surface (3). In either situation fatty acids cross the cell membrane, enter the cytosol, and are usually activated to an acyl-CoA derivative for subsequent metabolic events. An intracellular protein capable of binding fatty acids and referred to as Z protein (4), sterol carrier protein (5), or FABP[1] (6) has been proposed to function as the intracellular mediator of fatty acid transport. Thus an important factor determining the flux of fatty acid into or out of cells is the partitioning of fatty acid between extracellular protein (albumin), plasma membrane, and intracellular protein (FABP).

The association of fatty acids with albumin is generally understood (1, 2) although the precise molecular details are still under investigation (7, 8). A recent study has suggested that albumin-bound fatty acid enters hepatocytes by a receptor-mediated process which recognizes albumin (9), but it is also believed that movement of fatty acid between albumin and membrane is limited by the diffusion of free ligands in the aqueous phase (10). The kinetics and structural aspects of the interaction between FABP and fatty acid have not been as extensively studied and remain unclear, although recent studies elucidating the amino acid sequence of liver and intestinal FABP (11, 12) clearly will permit detailed structural studies to be performed.

Several studies have shown that long chain fatty acids will associate with phospholipid bilayers, and changes in the properties of the bilayer have been documented by different techniques, including calorimetry, nuclear magnetic resonance, and fluorescence (13–16). Relatively few studies have directly examined the movement of fatty acids into and out of membranes. Sengupta et al. (17) used fluorescence techniques to show that a pyrene derivative of decanoic acid transfers rapidly between lipid bilayers and the transfer followed first-order kinetics. Exchange between unilamellar vesicles occurred within seconds, whereas exchange was much slower using multilamellar structures. Doody et al. (18) examined in detail the transfer of 9-(3-pyrenyl)-nonanoic acid between synthetic unilamellar vesicles and demonstrated rapid exchange by a process that followed first-order kinetics with no change in rate over a wide range of donor and acceptor vesicles, suggesting that the transfer of fluorescent analog was mediated by diffusion in the aqueous phase, rather than by collision mechanisms. These studies indicated that transfer of the ionized fatty acid analog occurred at a more rapid rate than the protonated form and that transverse motion across a bilayer occurred faster than movement between bilayers. Hauser et al. (19) proposed that fatty acids exist as clusters within phopholipid bilayers and suggested that spin-labeled analogues of fatty acids effected bilayer structure differently than naturally occurring fatty acids.

In the present study we have developed a simple method for examining the movement of fatty acids from multilamellar liposomes to albumin or the hepatic FABP and have used this system as a model for studying several factors that might influence fatty acid transport across cell membranes.

EXPERIMENTAL PROCEDURES

Materials—Egg yolk phosphatidylcholine (Grade 1) was purchased from Lipid Products, Surrey, United Kingdom. Bovine serum albumin (essentially fatty acid free), dipalmitoylphosphatidylcholine, dimyristoylphosphatidylcholine, oleic acid, palmitic acid, and the respective sodium salts were obtained from Sigma. Oleoyl-CoA was purchased from P-L Biochemicals. [2-*palmitoyl*-9,10-³H]Dipalmitoyl phosphatidylcholine (60 Ci/mmol), [1-¹⁴C]oleic acid (56 mCi/mmol) and [1-¹⁴C]palmitic acid (58 mCi/mmol) were obtained from New England Nuclear. Sephadex G-25 (medium) was from Pharmacia. Liquiscint is a product of National Diagnostics (Somerville, NJ). All reagents used were of the highest grade available, and water was deionized and distilled in glass.

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[1] The abbreviation used is: FABP, fatty acid-binding protein.

Methyl [1-¹⁴C]oleate was synthesized by methylation in boron trifluoride-methanol of [1-¹⁴C]oleic acid (20). The methyl oleate was extracted into hexane, and the purity of the product was confirmed by thin-layer chromatography using hexane:ethyl ether:acetic acid (90:5:1) as the developing solvents. FABP was purified from livers of adult female rats exactly as described by Dempsey *et al.* (5). The purified material appeared as a single band on sodium dodecyl sulfate-polyacrylamide gel electrophoresis and migrated slightly faster than a horse myoglobin standard. The molecular weight was assumed to be 14,000 daltons. Aliquots of the purified material were stored at −70 °C in 0.03 M Tris, 0.4 M NaCl, pH 8.9, at concentrations between 250–400 µM.

Preparation of Liposomes—Liposomes were prepared by first dissolving the designated amount of lipid in chloroform:methanol (2:1 v/v), evaporating the organic solvent under a nitrogen stream, and then freeze-drying the lipid residue for 60 min under vacuum. The lipid was then resuspended in buffer with periodic gentle agitation for about 2 h at ambient temperature. Standardized conditions were established so that 1 ml of liposomes containing 40 mM lecithin, 13 mM cholesterol, and varying amounts of oleic acid were prepared in a buffer consisting of 0.01 M Tris, pH 7.4, 0.1 M NaCl, and 0.02% sodium azide (Tris-NaCl buffer). Tritiated lecithin and ¹⁴C-labeled fatty acid were routinely included in the liposome preparation using 1.0 µCi of each isotope.

Incubation of Liposomes and Protein—Reaction conditions were standardized so that aliquots of the stock liposome preparation were added to plastic microfuge tubes (total capacity, 400 µl), diluted with Tris-NaCl buffer, and an aliquot of stock protein solution was added to a final volume of 150 µl. The tubes were incubated under stated conditions and then centrifuged in a Beckman microfuge for 2 min (assumed centrifugal force of 10,000 × g). Aliquots (100 µl) of the resulting supernatant were removed and added to 4.0 ml of Liquiscint for measurement of radioactivity. Scintillation counting was performed using a Packard Tricarb 300 CD programmed to give a 19% crossover of ¹⁴C into the ³H channel. The amount of fatty acid present in the supernatant and bound to protein was calculated from the specific activity of the ¹⁴C-fatty acid in the original liposome preparation after correcting for the amount of unsedimented liposomes based on the [³H]phospholipid remaining in the supernatant.

Gel Filtration Binding Assay—Binding was measured by a modification of a gel filtration procedure described by Fry *et al.* (21). Sephadex G-25 swollen in Tris-NaCl buffer was added to 3-ml plastic syringes fitted with a 53-µm nylon mesh to a final packed volume of about 3 ml. The syringes were placed in test tubes (16 × 100 mm) and centrifuged for 3 min at 1000 × g in an IEC 7R centrifuge at ambient temperature. The syringe was then placed into a new test tube containing an empty 1.5-ml plastic microfuge tube. A 200-µl aliquot of sample containing labeled fatty acid and protein was removed for scintillation counting, and a second 200-µl aliquot was then applied to the partially dehydrated gel bed, and the syringe plus test tube was recentrifuged at 1000 × g for 3 min. The liquid centrifuged into the 1.5-ml microfuge tube was collected and analyzed for radioactivity or protein. Preliminary experiments established that the volume of fluid contained in the microfuge tube following the second centrifugation was within 5% of the volume of sample applied. When albumin was the sample applied, over 90% was routinely recovered, for FABP recovery averaged 80%, and if fatty acid without protein was applied to the column, all the labeled fatty acid was retained by the column at concentrations below 100 µM. Thus the procedure effectively separated protein from fatty acid.

Samples routinely contained 0–60 µM fatty acid added from a stock solution of 1 mM of the sodium salt pre-equilibrated with [1-¹⁴C]oleic or palmitic acid so that the final specific activity was about 7500 dpm/nmol. Prior to incubation, FABP was added to a final concentration of between 5–30 µM. For studies performed at different pH, the Sephadex column was equilibrated with the appropriate buffer prior to the first centrifugation, and the sample was applied in a solution prepared at the designated pH.

Sucrose density gradient ultracentrifugation was performed by layering 0.2-ml samples onto 4.0 ml of a 5–20% continuous sucrose gradient containing Tris-NaCl buffer. Centrifugation was performed for 16 h at 16 °C at 53,000 rpm using a Beckman SW 60Ti rotor. Fractions were collected from the bottom of the tube and analyzed for radioactivity or protein.

Phospholipid was measured by the method of Bartlett (22), protein was measured by the method of Lowry *et al.* (23), and for experiments using sucrose gradients, the dye binding procedure of Bradford was employed (24).

RESULTS

The general protocol used for these studies involved the preparation of liposomes containing varying amounts of labeled fatty acid, diluting these liposomes with a buffered solution either lacking or containing protein, and then separating the liposomes from the solution by rapid centrifugation to determine how much labeled fatty acid was protein bound. In preliminary experiments it was found that liposomes prepared with egg yolk lecithin and cholesterol at concentrations of 40 and 13 mM, respectively, and oleic acid at concentrations of 2 mM or less, when diluted at least 10-fold with buffer, would sediment in a Beckman microfuge after 2 min such that more than 95% of the lipid was pelleted. The molar ratios of nonsedimenting lipid were identical to the original molar ratio as determined by isotopic or chemical analysis. When samples were sedimented at greater centrifugal force (100,000 × g for 15 min) over 99% of lipid sedimented. When solutions of albumin or FABP were centrifuged either in the absence or presence of liposomes over 98% of the protein was in the supernatant. Liposome preparations were routinely examined in a polarizing light microscope and had the characteristic appearance of birefringent multilamellar liposomes.

Some properties of the assay system used are shown in Fig. 1. Fig. 1A shows the effect of incubation time on the movement of fatty acid from liposomes to protein using reaction conditions when liposomes containing lecithin:cholesterol:oleic acid (molar ratio 100:33:5) were diluted 20-fold and incubated with either 10 µM albumin or FABP. The fatty acid rapidly associated with protein, and an apparent equilibrium was reached within 15 min when FABP was present. Incubation with albumin resulted in about 35% of the total fatty acid present originally (100 µM) to be protein bound, corresponding to a fatty acid-protein molar ratio of about 3.5:1. When FABP was added, the apparent equilibrium was reached at fatty acid to protein molar ratio of about 1. The effect of protein concentration is shown in Fig. 1B where liposomes were incubated for 60 min with varying amounts of protein. FABP consistently bound approximately equimolar amounts of oleate whereas albumin binding was more complex. In similar experiments with albumin where incubation time and fatty acid concentration relative to lecithin were varied, molar ratios of bound oleate to albumin up to 7:1 were obtained, and up to 75% of the total fatty acid present could be removed from the liposomes by albumin.

The movement of fatty acid from liposomes to protein was a reversible process. Fig. 1C shows results of experiments where equimolar amounts of oleate and protein were allowed to equilibrate for 1 h and then incubated with varying amounts of liposomes containing lecithin and cholesterol but no fatty acid. Following incubation at 25 °C for 16 h, the distribution of labeled oleate between liposomes and protein was determined and found to be dependent on the relative amount of liposomes added using either albumin or FABP. Separate experiments showed that movement of fatty acid from protein to liposomes was a much slower process than the opposite effect (data not shown). Several experiments were performed where protein was incubated with liposomes containing [4-¹⁴C]cholesterol and [³H]lecithin to assess the effects of protein on the sedimentation of the major lipid components. The distribution of either phospholipid or cholesterol between pellet and supernatant was unaffected by incubation time. Fig. 1D shows that addition of protein did have a small but definite effect in that albumin actually decreased the per cent
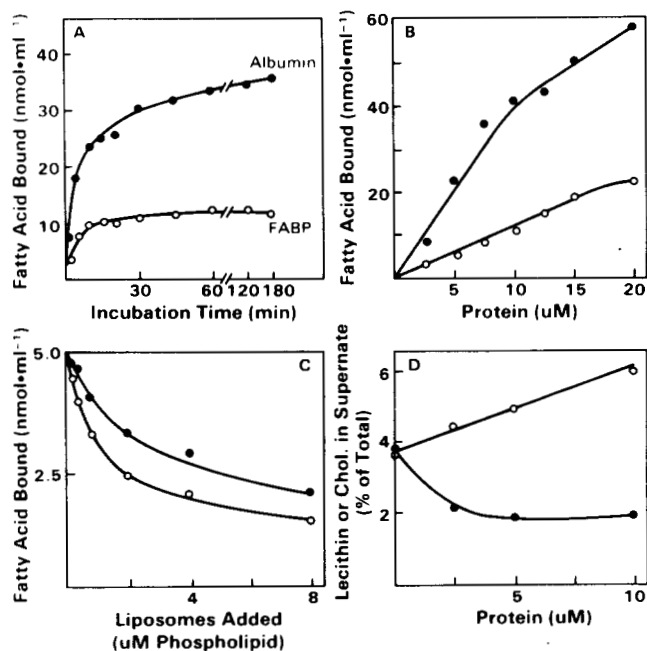
FIG. 1. **Characteristics of the assay used to measure fatty acid transfer between liposomes and albumin (●) or FABP** (○). All incubations were performed at 25 °C. Following incubation, the reaction mixture was centrifuged, and the labeled oleate in the supernatant was determined as described under "Experimental Procedures." *A*, effect of incubation time. Liposomes (molar ratio, 100:33:5) were diluted to an oleic acid concentration of 100 μM and incubated for different times with either 10 μM albumin or 10 μM FABP. *B*, effect of protein concentration. Liposomes were diluted to 100 μM oleic acid concentration and incubated for 60 min with varying amounts of albumin or FABP. *C*, either albumin or FABP were initially mixed with an equimolar amount of labeled oleate and diluted to 10 μM in reaction mixtures with varying amounts of liposomes containing egg lecithin and cholesterol but not fatty acid incubated for 60 min. *D*, effect of albumin or FABP on the sedimentation of labeled lecithin and cholesterol in liposomes containing unlabeled oleate (molar ratio, 100:33:5) diluted 20-fold.

of total phospholipid or cholesterol remaining in the supernatant from a control level of 4 to about 2%, whereas FABP caused an increase to about 6%. No difference in the relative distribution of phospholipid and cholesterol was ever observed, indicating that under the experimental conditions employed neither albumin nor FABP significantly influenced the multilamellar nature of the liposomes.

Reaction mixtures containing liposomes and either albumin or FABP also were analyzed by sucrose density gradient ultracentrifugation (Fig. 2, *A* and *B*). When albumin was present (Fig. 2*A*) about 60% of the total fatty acid present in the reaction mixture sedimented into the gradient and appeared exactly where albumin migrated. Phospholipid was found at the top of the gradient together with nonprotein-bound fatty acid, but no phospholipid was associated with protein. Analogous results were obtained using FABP, which sedimented less rapidly than albumin (Fig. 2*B*). Sucrose gradients also were used to show the binding of oleate to FABP in the absence of liposomes. Fig. 2*C* shows that when equimolar amounts of labeled oleate and FABP were analyzed, both protein and fatty acid co-sedimented into the gradient. Fig. 2*C* shows data for a 5 μM mixture of fatty acid and protein, but qualitatively identical results were found using 50 μM FABP, with or without fatty acid, suggesting that FABP does not aggregate over this concentration range.

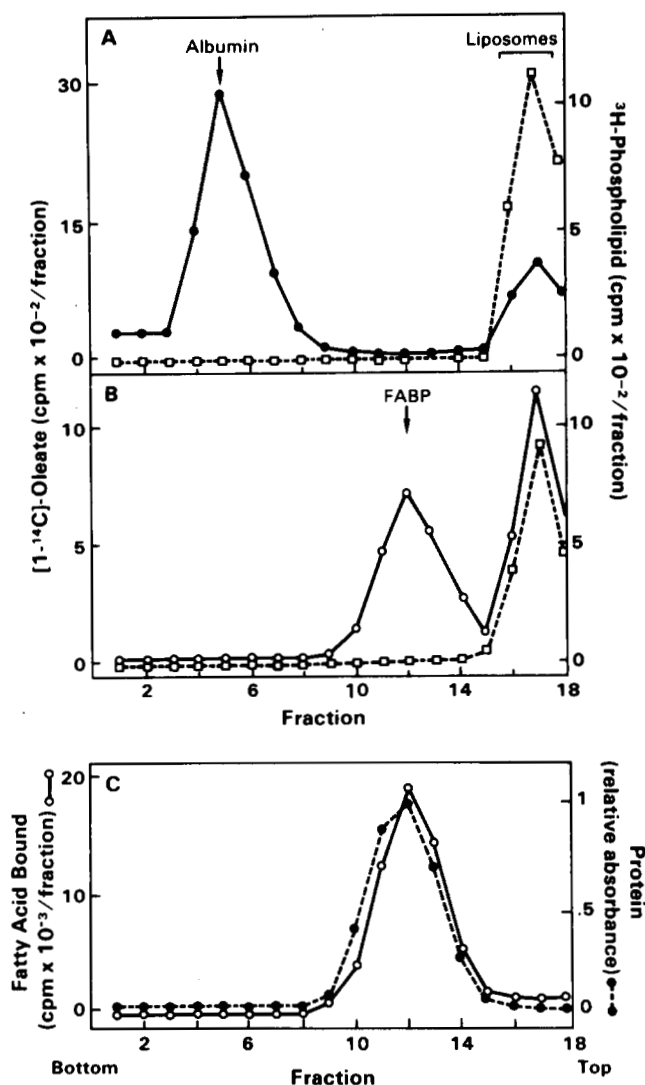Fig. 3 shows data where different amounts of liposomes

FIG. 2. **Sucrose density gradient ultracentrifugation of mixtures containing liposomes, labeled oleate, and protein.** Liposomes (molar ratio, 100:33:5) were diluted to an oleic acid concentration of 50 μM and incubated with 10 μM albumin or FABP for 60 min at 25 °C. 0.2 ml of the reaction mixture was applied to 5–20% sucrose gradients. *A*, albumin and liposomes; *B*, FABP and liposomes; *C*, 5 μM FABP was incubated with 5 μM labeled oleate for 30 min at 25 °C, and 0.2 ml was applied to the gradient.

(molar ratio 100:33:5) were incubated with protein for 16 h at 25 °C to ensure complete equilibration. The amount of protein-bound fatty acid is plotted relative to the total concentration of oleic acid in the incubation. Using 5 or 10 μM FABP, the apparent saturation occurred when equimolar amounts of oleate were bound to protein. Using 8 μM albumin, saturation was reached at a 4:1 molar ratio. The *inset* of Fig. 3 is a Scatchard plot of the data for FABP at 5 and 10 μM and suggests a single binding site with a K_d of about 12 μM. Similar experiments using a shorter incubation time of liposomes with less fatty acid relative to lecithin gave apparent K_d values ranging from 10–18 μM. Use of the Scatchard plot in Fig. 3 assumes that all the fatty acid originally associated with liposomes was available for binding. As an independent test of this assumption, binding of oleate to FABP was determined in the absence of liposomes using a modification of the method of Fry *et al.* (21) to separate bound and free oleate. Fig. 4 shows a representative experiment where 5 μM FABP was incubated for 60 min at 25 °C with varying amounts of
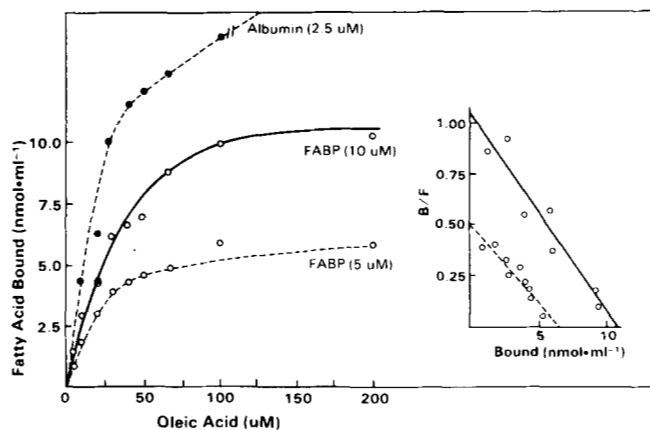
FIG. 3. **Effect of fatty acid concentration on the movement of oleic acid from liposomes to protein.** Varying amounts of liposomes (molar ratio, 100:33:5) were incubated with albumin or FABP for 16 h at 25 °C. Reaction mixtures were centrifuged and the labeled oleate in the supernatant determined. *B/F*, bound/free.
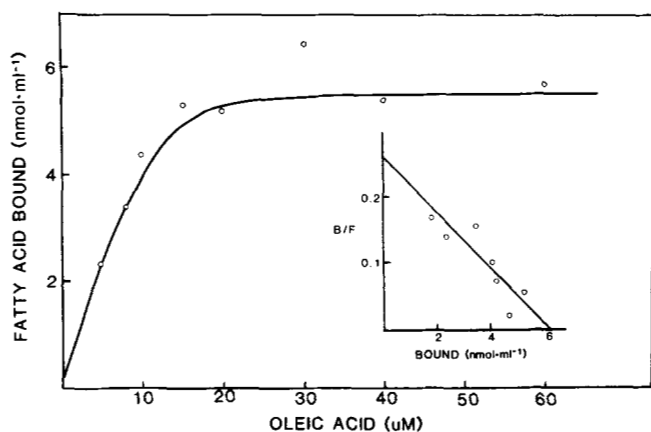

FIG. 4. **Binding of oleic acid to FABP as determined by gel filtration through Sephadex G-25 minicolumns.** Varying amounts of sodium oleate (specific activity, 7950 dpm/nmol) were incubated with 5 μM FABP for 1 h at 25 °C, and then 0.2-ml aliquots were applied to partially dehydrated Sephadex minicolumns. Following centrifugation, the amount of labeled oleate bound to FABP was determined by measuring radioactivity and protein in the eluate. *B/F*, bound/free.

FIG. 5. **Effect of pH on fatty acid and methyl oleate binding to albumin or FABP.** *A,* liposomes were prepared containing oleic acid (molar ratio, 100:33:5) or methyl oleate (molar ratio, 100:33:1) and diluted 20-fold into buffers of the designated pH containing either 10 μM albumin (●) or 10 μM FABP (○). Following incubation for 60 min at 25 °C, samples were centrifuged, and the labeled oleate or methyl oleate in the supernatant was determined. *B,* equimolar mixtures of sodium oleate or methyl oleate and either albumin or FABP were preincubated for 60 min at 25 °C and then diluted to final concentration of 10 μM in the designated buffer. Aliquots were applied to Sephadex minicolumns, and bound ligand was determined as described under "Experimental Procedures."

labeled oleate. Saturable binding was observed at approximately equimolar amounts of fatty acid and protein, and the Scatchard plot suggested a K_d of 6 μM. In similar experiments where incubation time or protein concentration was varied, K_d values between 5 and 12 μM were obtained with molar ratios of fatty acid and protein at saturations ranging between 1 and 1.5.

The influence of pH on the movement of fatty acid from liposomes to protein was studied using liposomes (molar ratio 100:33:5) that were formed in the standard buffer and then diluted 20-fold into buffers of varying pH prior to incubation with protein. Fig. 5A shows that binding to either albumin or FABP was reduced as the pH was lowered to 6, and virtually no binding occurred at pH 4.0. This effect could be due either to the impaired association of fatty acid to protein at low pH or a reduced dissociation of fatty acid from the liposomes. Fig. 5B shows the effect of pH on binding of oleate to protein in the absence of liposomes using the gel filtration procedure. Binding to both proteins was reduced as the pH was decreased but not to the extent observed using liposomes as the source of fatty acid. For example, FABP bound oleate almost as well
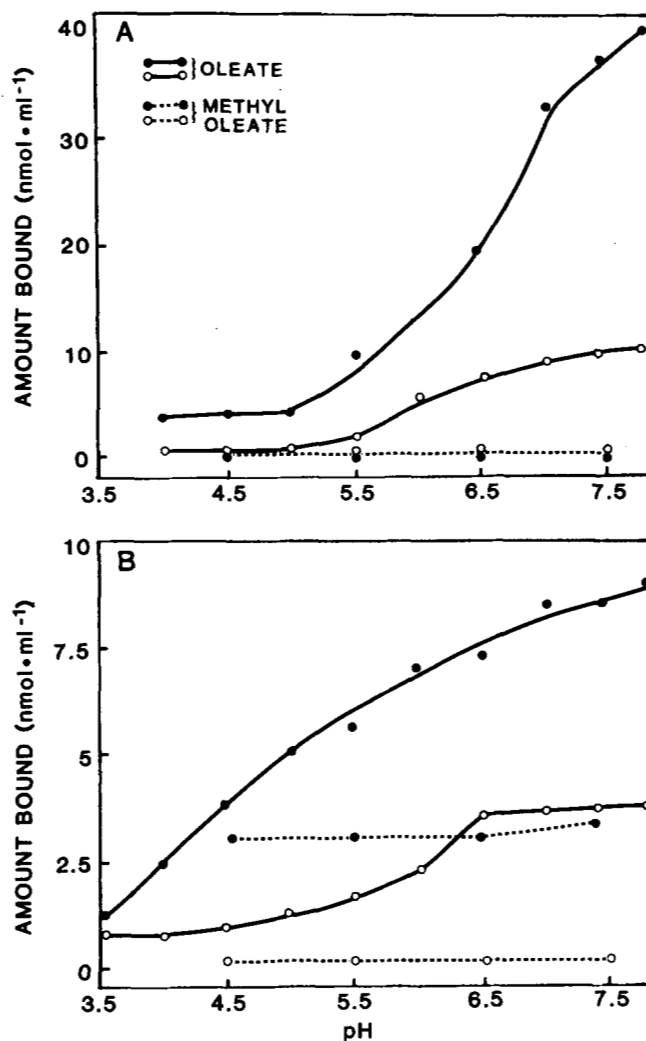
at pH 6 and 7.5, and binding at pH 4 was about 50% of that observed at pH 7.4. Direct measurement of the radioactivity associated with the liposome pellet in the experiment in Fig. 5A showed that the fatty acid was in the pellet and had not been artifactually adsorbed to the walls of the centrifuge tubes. Virtually all the protein was recovered in the supernatant both at low and high pH.

To further assess the possible influence of the ionization state of the fatty acid on movement from liposomes to protein, labeled methyl oleate was incorporated into liposomes or added directly to protein. As shown in Fig. 5A, methyl oleate did not move from liposomes to either protein at any pH studied. When methyl oleate was added directly to albumin in equimolar amounts, binding was observed over a wide pH range as determined by the gel filtration procedures (Fig. 5B). When similar experiments were performed using FABP no binding occurred, an observation that was not surprising since
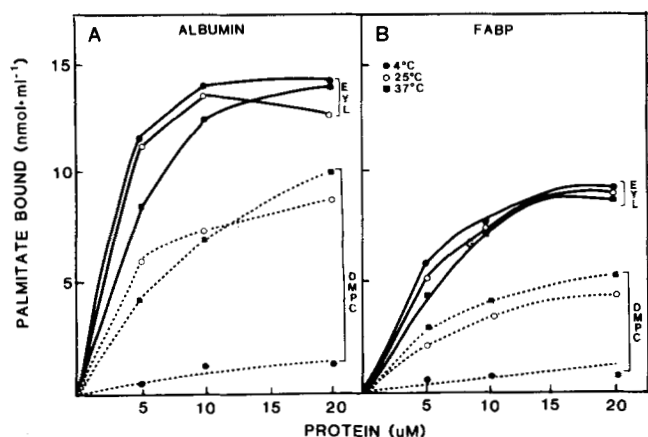
FIG. 6. **Effect of temperature on the movement of palmitic acid from egg yolk lecithin (*EYL*) liposomes or dimyristoyl-phosphatidylcholine (*DMPC*) liposomes to protein.** Liposomes were prepared at molar ratios of 100:1 and at a temperature of 30 °C. Liposomes were diluted 20-fold (palmitate concentration = 20 μM) and incubated with protein at the designated temperature for 60 min. *A*, albumin; *B*, FABP.

FABP was first characterized as protein binding organic anions.

To determine whether the physical state of the liposomes might influence the accessibility of fatty acid to the protein, comparative experiments were performed using liposomes made from dimyristoyl lecithin or egg yolk lecithin combined with palmitic acid at molar ratios of 100:1. Cholesterol was omitted from these liposome preparations. Fig. 6, *A* and *B*, shows that palmitate binds to either albumin or FABP between 4 and 37 °C when egg yolk lecithin was the phospholipid used and temperature had little quantitative effect. Using dimyristoyl lecithin, binding was observed to both proteins at 37 and 25 °C but was markedly less at 4 °C. Fig. 7 shows more convincingly the effect of temperature on the movement of palmitic acid from liposomes to protein. In these experiments liposomes containing palmitic acid and either dimyristoyl lecithin or dipalmitoyl lecithin (molar ratio 100:1) were prepared and stored at 35 and 55 °C, respectively, and then incubated with 10 μM protein at the designated temperature for a relatively brief incubation time (10 min). Decreased binding was clearly observed at temperatures corresponding to the known transition temperatures for those phospholipids. To ensure that the small amount of palmitate present in the liposomes did not affect the physical state of the bilayer appreciably, differential scanning calorimetry of the dipalmitoyl lecithin preparation was performed[2] and the results (not shown) indicated a major transition at 42 °C and a pretransition at 35 °C, a feature characteristic of dipalmitoyl lecithin liposomes (14). The possibility that the proteins might associate with the liposomes at temperatures below the transition was ruled out by separate experiments showing that most of the total protein added was in the supernatant following incubation with either preparation at temperatures above and below the expected transition.

DISCUSSION

The use of multilamellar liposomes provided a convenient experimental system to study certain factors regulating the movement of long-chain fatty acids across membranes. An

[2] Calorimetry was performed with a Perkin-Elmer DSC2 differential scanning calorimeter with the assistance of members of the Biophysics Section (Dr. Donald M. Small, Director).
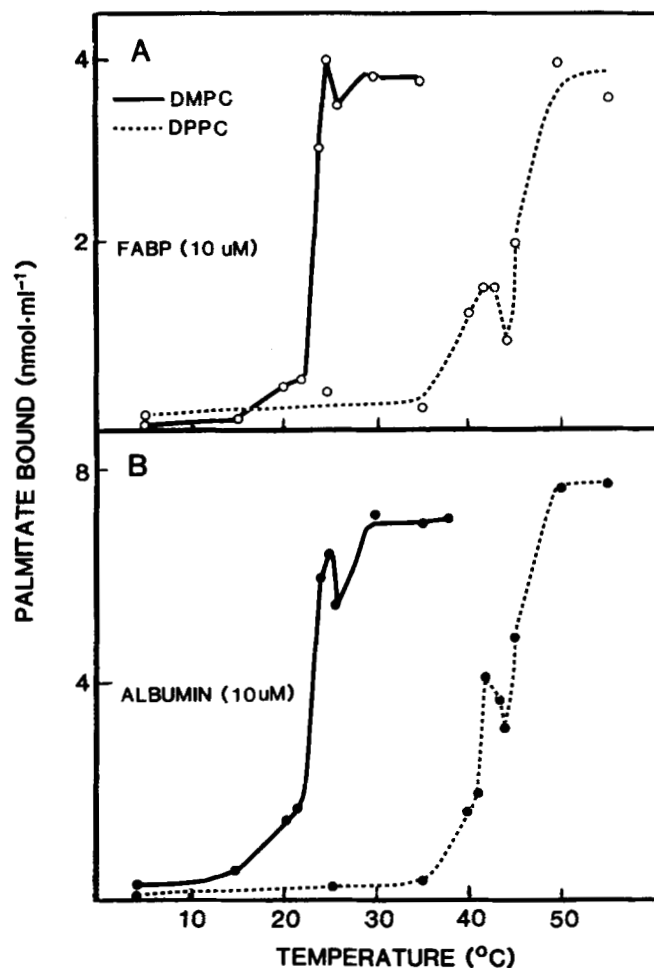

FIG. 7. **Effect of temperature on the movement of palmitic acid from dipalmitoylphosphatidylcholine (*DPPC*) liposomes or dimyristoylphosphatidylcholine (*DMPC*) liposomes to protein.** Liposomes were prepared at molar ratios of 100:1 at temperatures of 35 °C for dimyristoylphosphatidylcholine and 55 °C for dipalmitoylphosphatidylcholine and stored at those temperatures until immediately prior to incubation. Liposomes were diluted 20-fold and incubated for 10 min at the designated temperature. *A*, albumin; *B*, FABP.

important observation was that when proteins capable of binding fatty acids were incubated with liposomes, more than 50% of the fatty acid contained in the liposome could be transferred to the protein under appropriate conditions. If it is assumed that the proteins do not disrupt liposomes and that only fatty acid molecules on the outer leaflet of the outermost bilayer of the liposome are exposed to protein, then removal of 50% of the fatty acid indicates that these molecules can diffuse readily between and across the concentric bilayers of the liposomes. Using fluorescent fatty acids evidence for movement between unilamellar vesicles was reported and it was suggested that transbilayer "flip-flop" was more rapid than movement between vesicles (18).

The mechanism for the movements of fatty acid from a bilayer to protein could involve a collision of protein with the outer leaflet of the liposome followed by a specific interaction with the fatty acid contained in the bilayer and ultimately a dissociation of the newly formed fatty acid-protein complex from the bilayer surface. Alternatively, fatty acid could desorb from the surface of the bilayer into the aqueous space and subsequently associate with protein by a diffusion-mediated process. Studies on the spontaneous transfer of cholesterol or

phospholipid between bilayers have shown clearly that dissociation of the monomeric lipid moieties from the bilayer into water is the rate-limiting step, and a "collision" mechanism has been ruled out by kinetic experiments (25, 26). In the case of fatty acids, it seems likely that desorption from the bilayer occurs much more rapidly than that for either cholesterol or phospholipid. The experimental system used in the present study was not suitable for determining reliable estimates of the initial rates of transfer, and, therefore, we could not calculate rate constants required to distinguish between collision or aqueous diffusion mechanisms. Nevertheless, these studies do show that fatty acid transfer does occur, and the process could be influenced by pH and the physical state of the liposomes.

The effect of pH on fatty acid movement from liposomes to protein suggests that the protonated form of fatty acid is tightly associated with the bilayer and not accessible to soluble proteins capable of fatty acid binding. The pK_a of fatty acid contained within phospholipid bilayers has been estimated using techniques based on electrophoretic mobility (19), nuclear magnetic resonance (27, 28), calorimetry (29), and fluorescence (18), and there seems to be general agreement only in that the pK_a is higher than that of the corresponding fatty acids in solution. Using fluorescent fatty acid the rate of transfer between bilayers was reported to be greater for the ionized than the protonated form (18). Since marked changes in the movement of oleic acid from liposomes to either albumin or FABP were observed over a pH range where the net charge on the lecithin would not change, it seems reasonable to assume that the ionization state of the fatty acid determines its partition between the bilayer and the aqueous environment. The lack of movement of the uncharged, less polar, and less water-soluble methyl ester over a broad pH range is consistent with the concept that the partition is determined, at least in part, by the solubility of the ligand in water.

A physiological implication of these observations on pH relates to the movement of fatty acids across the lysosomal membrane. Fatty acids are generated within the acidic environment of the lysosome by lipases and phospholipases, and in order to leave the organelle the fatty acid, presumably at least partially protonated, must associate with the inner leaflet of the bilayer, traverse the bilayer, and then dissociate from the cytosolic side, probably in the ionized form. Our data suggest that the pH gradient across the lysosomal membrane would direct the flux of fatty acid out of the lysosome into the cytosol. The presence of FABP in the cytosol may maintain this flux if the protein-bound fatty acid were directed toward other organelles.

In our studies on the effect of temperature we used liposomes with only 1 mol per cent of palmitic acid, and cholesterol was omitted to ensure the transitions characteristics of the phospholipids would not be altered. Several studies (14, 15, 29, 30) have shown that relatively high concentrations of fatty acids (more than 10 mol %) did cause changes in the thermal properties of liposomes, including the elimination of the pretransition, but we observed no major changes in the liposomes used in the present study. Furthermore, Hauser *et al.* (19) reported that incorporation of up to 10 mol % stearic acid into phosphatidylcholine dispersions did not alter the general properties of the multilamellar liposomes.

The effects of temperature on fatty acid movement from liposomes to protein show clearly the importance of the physical state of the phospholipid. Binding occurred readily at all temperatures tested when egg yolk lecithin liposomes were used, and it is known that this phospholipid is in the liquid-crystalline state at temperatures above −10 °C. The marked differences in protein binding above and below the reported transition temperatures for dimyristoyl lecithin and dipalmitoyl lecithin (31) suggest that either palmitate does not readily diffuse out of bilayers that are in the gel state or that the proteins cannot facilitate the removal of fatty acid from the bilayer. The irregular change in protein binding observed with both proteins close to the transition temperatures may reflect enhanced dissociation of fatty acid occurring at the interface between gel and liquid-crystalline domains, both of which are thought to exist at temperatures near the transition points (32). Alternatively, either protein could selectively associate with such regions and facilitate removal from the bilayer. The existence of such domains in biological membranes (33) raises the possibility that fatty acid transport across bilayers may be influenced by localized changes in membrane composition or fluidity.

In these studies we have used albumin and hepatic FABP as examples of extracellular and intracellular fatty acid-binding proteins. The physiological role for albumin is clear, but the mechanism involved in the movement of fatty acid between albumin and cell membranes is controversial. Evidence for an albumin receptor on the cell surface of hepatocytes has been reported (9), a mechanism for permeation of long-chain fatty acids into adipocytes involving a facilitative transport by a phloretin-inhibitable process was proposed (34), and the possibility that albumin may overcome diffusion barriers at cell surfaces was discussed (10). During lipolysis of triacyclglycerol contained in lipoproteins at the surface of endothelial cells, it was suggested that albumin may not be involved in fatty acid transfer from plasma to cell membranes (3).

Evidence for FABP as a major intracellular protein involved in the transport of fatty acid, although compelling (12, 35), remains circumstantial. The techniques we used to demonstrate fatty acid binding to FABP indicated that saturable binding occurs at equimolar amounts, but the apparent K_d values differed. This is not surprising since when liposomes are present, at equilibrium the fatty acid is partitioning between the liposomes and proteins, whereas in the absence of liposomes, fatty acid will either be bound to protein or present as monomers or aggregates. In the latter case the gel filtration method used to separate bound and unbound fatty acid could perturb actual equilibrium conditions causing the estimated apparent K_d to be higher than the true values. Our findings are consistent with previous reports showing a single fatty acid-binding site for FABP (36, 37) and suggest that most of the fatty acid contained within liposomes has access to the outer leaflet of the multilamellar structure which is exposed to the protein. Furthermore, the data show that FABP can effectively remove fatty acids from synthetic bilayers by a mechanism similar to that of albumin, suggesting that these proteins could have a major role in regulating the flux of fatty acids across biological membranes.

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