Perspectives in Diabetes

How Are Free Fatty Acids Transported in Membranes?

Is It by Proteins or by Free Diffusion Through the Lipids?

James A. Hamilton and Frits Kamp

Although transport of long-chain free fatty acids (FFAs) into cells is often analyzed in the same way as glucose transport, we argue that the transport of the lipid-soluble amphipathic FFA molecule must be viewed differently. The partitioning of FFAs into phospholipid bilayers and their interfacial ionization are particularly relevant to transport. We summarize new data supporting the diffusion hypothesis in simple lipid bilayers and in plasma membranes of cells. Along with previous supporting data, the new data indicate that transport of FFAs through membranes could occur rapidly by flip-flop of the un-ionized form of the FFA. It appears that, at least for the adipocyte, passive diffusion guarantees fast entry and exit of FFAs at both low and high concentrations. Although there are several candidate proteins for the membrane transport of FFAs, most of these proteins have other established functions. Thus, unlike the glucose transporters, these proteins would not be single-function proteins. Definitive proof of their function as FFA transporters awaits their reconstitution into simple model systems. *Diabetes* 48:2255–2269, 1999

How does fatty acid transport compare with glucose transport? Long-chain nonesterified or "free" fatty acids (FFAs) and glucose are fundamental units of key nutrients for human cells. Both are present in blood and need to move from the plasma compartment into cells for utilization. Both are oxidized immediately to provide fuel for sustenance of life (particularly in muscle cells) or are stored as larger molecules (triglycerides or glycogen). When intracellular stores are metabolized, the released FFA or glucose is often exported from the cell for utilization at another site. A significant portion of the FFA taken up by a cell may also be used for synthesis of other acylated lipids, such as phospholipids and cholesteryl esters, and a minor fraction for synthesis of prostaglandins and other potent signal agents. Similarly, a minor fraction of glucose may be used for synthesis of glycosylated proteins or lipids within the cell.

The fasting concentration of glucose in blood is tightly regulated (~5 mmol/l) (1) by insulin and other hormones, by membrane transporters, and by metabolism, because both high and low concentrations are damaging or toxic. Glucose and FFA uptake are sometimes linked. For instance, insulin stimulates uptake of both glucose and FFAs into adipocytes by providing the glycerol backbone through stimulation of glycolysis. Additional mechanisms might account for insulin-stimulated FFA uptake (2). For instance, acyl-CoA synthase, the enzyme that activates FFAs for metabolism (esterification or β-oxidation), is associated with GLUT4 vesicles in adipocytes (3). Thus, when GLUT4 vesicles bind with the plasma membrane, which is stimulated by insulin, intracellular activation and trapping of FFAs might be enhanced as well as glucose transport.

The venous blood concentration of FFAs is known to vary widely (~0.25–3.0 mmol/l), depending on nutritional status and exercise, and is chronically higher in individuals with obesity and/or diabetes (4,5). In the capillaries and the microenvironments of cells, the concentration of FFAs is likely to be higher and to vary even more than revealed by an analysis of venous blood. For example, much of the FFA that enters a cell is derived from lipolysis of triglycerides contained in circulating lipoprotein particles, and this reaction is likely to produce a very high (transient) local concentration in the capillary endothelium. Although difficult to measure, such quantities need to be considered for both normal and abnormal cell physiology. In contrast, since polysaccharides are converted to highly water-soluble glucose molecules before entering the blood, glucose is probably homogeneously distributed throughout the vascular system. In spite of the limitation of measuring only the venous blood level of FFAs (which is almost entirely bound to albumin), the chronically high levels of FFAs in the blood (and the higher FFA:albumin ratio) appear to be intimately if not mechanistically linked to the onset of type 2 diabetes (2,6–8).

From the Departments of Biophysics and Medicine, Boston University School of Medicine, Boston, Massachusetts.

Address correspondence and reprint requests to Dr. James A. Hamilton, Departments of Biophysics and Medicine, Boston University School of Medicine, 715 Albany St., Boston, MA 02118. E-mail: hamilton@med-biophd.bu.edu.

Received for publication 18 August 1999 and accepted in revised form 30 September 1999.

BSA, bovine serum albumin; FABP, fatty acid binding protein; FABPpm, fatty acid binding plasma membrane protein; FAT, fatty acid transporter; FAT1, fatty acid transporter 1; FATP, fatty acid transport protein; FFA, free fatty acid; $[FFA]_a$, concentration of unbound monomeric free fatty acid in the water phase; k_{off}, off-rate constant; k_{on}, on-rate constant; K_p, partition coefficient; LUV, large unilamellar vesicle; NMR, nuclear magnetic resonance; OA, oleic acid; PA, palmitic acid; pH_{in}, internal pH; SUV, small unilamellar vesicle; UCP, uncoupling protein; ν, moles of FFA bound to albumin/moles of albumin; VLCFA, very-long-chain fatty acid.

Although it is not clear whether the blood levels of FFAs are regulated in a manner similar to glucose, many investigators conceptualize the transport and uptake of FFAs into cells in the same way as the transport of glucose (9–12). For example, as is illustrated in Fig. 1*A*, glucose uptake into erythrocytes plotted against the concentration of glucose in the external buffer shows saturation. The limitation of transport can be understood in a straightforward manner as saturation of the binding sites for glucose on the transport protein, without which there is very little uptake of glucose (Fig. 1*A*), i.e., simple diffusion is not effective for moving glucose through the membrane. Similarly, experimental data for the uptake of FFAs into cells is often quantitated by the relationship between external unbound FFA ($[FFA]_a$) and the initial uptake rate of FFA by the cells, and this relationship sometimes has a "saturable component" (Fig. 1*B*). The saturation effect has long been one of the keystones of the hypothesis of protein-mediated membrane transport of FFAs (9,10,13). Also in analogy to the case of glucose, it has been suggested that electrical stimulation of cells might translocate FFA transporters from intracellular sites to the plasma membrane (10). It has also been hypothesized that a transporter analogous to GLUT2 must mediate cellular FFA efflux after intracellular lipolysis of triglycerides (14).

Thus, whether implicitly or explicitly, transport of typical dietary (long-chain) FFAs through membranes is often framed in the context of glucose transport. However, the molecular properties of glucose and FFAs are quite different. Long-chain FFAs are very water insoluble. Because of their polar headgroup and hydrophobic tail, FFAs interact with membrane lipids in a specific way. The same is not true for glucose, which is very water soluble and has a low solubility in membrane lipids (15). Therefore, the model described in Fig. 1*A*, in which the transported molecule binds only to a protein and is excluded from the lipid phase, is applicable to glucose but not to FFA, and a much different paradigm for FFA transport might be envisioned. The general issue of FFA transport through membranes has been controversial for many years. Some investigators hypothesize that FFA transport is a simple diffusion process according to the classical paradigm for short- and medium-chain FFAs (13,16–20). Others have proposed a more complicated process involving one or more proteins (10–12,21,22). It has also been suggested that at low FFA concentrations, FFAs are transported by high-affinity transport proteins, and at high FFA concentrations, FFAs move across the plasma membrane predominantly by free diffusion (Fig. 1*B*) (10,23,24).

A better understanding of the mechanisms of FFA transport is essential for identifying the mechanism(s) by which FFAs cause or exacerbate insulin resistance and diabetes. Do FFAs accumulate outside a cell until signal(s) begin a process to bring transport proteins to the plasma membrane to allow FFAs to enter a cell? Are certain FFAs selected at the membrane for entry into the cell? Can FFAs desorb from the inner leaflet of the plasma membrane and diffuse to all parts of the cell or is their intracellular transport dependent on cytosolic transporters? In this perspective, we discuss the physicochemical properties of FFAs and the biophysical concepts for FFA transport in simple well-controlled model systems. We show that key properties of FFA transport can be illuminated in simple model systems, and that predictions from models can be tested in living cells. We then discuss new data from studies of FFA transport in cells and speculate on what role proteins may play in FFA uptake in the context of known properties of FFAs.

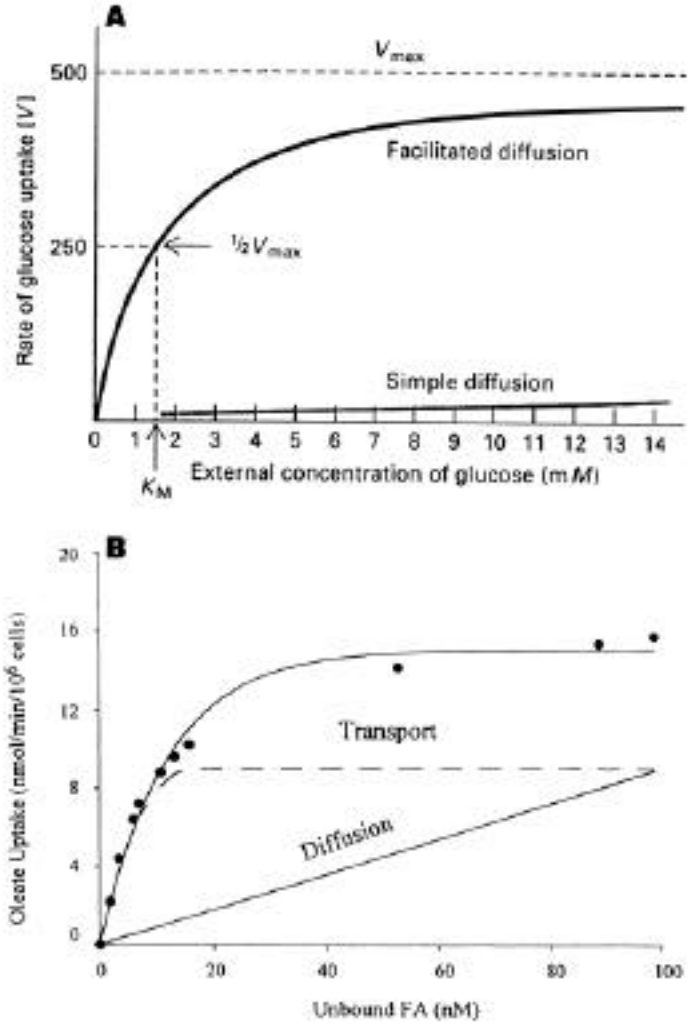

FIG. 1. *A*: The transport rate of glucose into erythrocytes as a function of glucose in the extracellular medium. The initial internal concentration is 0.5 mmol/l. The external glucose concentration is increased instantaneously. As a measure of facilitated diffusion (i.e., protein-mediated), the rate of glucose uptake in the first few seconds upon imposing various concentration gradients is plotted against the external glucose concentration. The lower line is the calculated curve for glucose uptake if this occurred solely by simple free diffusion. The measured curve displays much higher uptake rates at all glucose concentrations where a maximum uptake rate is saturable. Reprinted from Darnell et al. (129). *B*: Saturable and nonsaturable components of the uptake of OA in isolated rat adipocytes. The unbound FFA concentrations were calculated according to Richieri et al. (34). The FFA:BSA ratio ranged from 0.25 to 2.0. The top curve represents total uptake (which is not very different in shape from the curve in *A*). The bottom curve reflects the estimated diffusion component, and the dashed curve shows the estimated protein-mediated FFA transport. It is not clear how the two components were distinguished quantitatively. It was concluded that at low unbound FFA concentrations, FFA transport is mediated by proteins, whereas at high concentrations, the free diffusion predominates. Reprinted from Abumrad et al. (10).

TABLE 1
Physical properties of two long-chain FFAs: PA and OA

	Solubility limit in water (μmol/l)	K_p to membranes	K_a to first three high-affinity binding sites of albumin	K_a to adipocyte FABP
PA	4	0.6×10^6	153; 122 and 75×10^6	15.0×10^6
OA	6	0.5×10^6	127; 100 and 40×10^6	21.2×10^6

The solubility limit (32), the K_p between water and egg phosphatidylcholine bilayers (33), and the association constant (K_a) to BSA (34) and to adipocyte FABP (33) were determined with the fluorescent FFA indicator ADIFAB. The relative error of these values depends on the accuracy of the determination of the probe parameters of ADIFAB that relate fluorescence to $[FFA]_a$ (32,128).

AQUEOUS SOLUBILITY OF FFAS AND THEIR BINDING TO ALBUMIN AND MEMBRANES

Aqueous solubility of FFAs is very low. Whereas glucose can be conveniently added to cells in vitro, long-chain (>12 carbons) FFAs present numerous difficulties for investigators, both in experimental design and in interpretation of data. Long-chain FFAs are water soluble only at very high pH values (>10), where they form micelles (25). Above their solubility limit at neutral pH, they form insoluble acid soaps, i.e., bilayers similar to phospholipid bilayers but with a mixture of ionized and un-ionized headgroups at the water interface (26,27). Below the solubility limit or in equilibrium with acid soaps, FFAs are monomeric or possibly very small easily dissociable dimers (28). As an example of the complications in working with FFAs, the solubility *limit* of a long-chain FFA is *1*) difficult to measure, *2*) usually determined indirectly, *3*) dependent on pH, and *4*) the subject of continuing controversy (29). For example, the solubility limit of palmitic acid (PA) (C16:0) at pH 7.4 has been reported to be as low as 0.1 nmol/l (30) and as high as 10 μmol/l (29,31). A recent estimate using the fluorescent fatty acid binding protein (FABP) probe ADIFAB gave a solubility limit of 4–16 μmol/l (32). ADIFAB has been used to determine the solubility limit of various FFAs (32), the partition coefficient (K_p) of FFAs between water and phospholipid bilayers (33), the equilibrium binding constants of FFA to albumin (34) and FABPs (33), and the unbound FFA concentrations in human serum (35) (see Table 1).

Binding of FFA to albumin. The much higher concentrations of FFAs in the blood (0.2–3 mmol/l) cannot be transported in the form of acid-soap aggregates. They are transported mainly in complexes with albumin (16). The very small fraction that is bound to plasma lipoproteins is probably slightly increased in diabetes (4). In addition, large quantities of FFAs are transported from the liver and intestine in the form of triglycerides (in lipoproteins) and are released as FFAs in the capillary endothelium at sites of utilization. Albumin (bovine serum albumin [BSA] and human serum albumin) has three high-affinity binding sites for FFAs and is a buffer for FFAs in solution ($[FFA]_a$) so long as the albumin concentration exceeds 0.5 μmol/l (34); in the plasma, its concentration is ~0.5 mmol/l. The relationship between the concentration of unbound monomeric FFA in the water phase, $[FFA]_a$, and the FFA:albumin molar ratio (ν) has been determined by multiequilibrium analysis using ADIFAB (34). When $\nu < 3$, the $[FFA]_a$ is <50 nmol/l for oleic acid (OA) or PA, ~20 times lower than found earlier (31).

Equilibrium of FFAs between water and membranes. Because of their low aqueous solubility, and their high solubility in lipids, FFAs partition readily into phospholipid bilayers. This can be expressed quantitatively as the partition coefficient

$$K_p = [FFA]_m/[FFA]_a = \{([FFA]_t - [FFA]_a)/[FFA]_a\}/(V_m/V_a)$$

where $[FFA]_m$ is the FFA concentration in the membrane, based on the volume of the membrane (V_m), and $[FFA]_t$ and $[FFA]_a$ are the total and unbound FFA concentrations based on the volume of the water phase (V_a). For a phospholipid bilayer, V_m/V_a has been calculated as 10^{-3}/mmol/l phospholipid (36). The K_p depends on the chain length and unsaturation of the FFAs and on the phospholipid composition of the bilayer (33,37,38) and is in the order of 1×10^6 for long-chain FFAs (Table 1). Similar values have been found for the partitioning of FFAs between water and the lipid phase of entire cells (39).

Partitioning of FFAs between albumin and membranes. In aqueous mixtures of albumin and membranes, FFAs generally partition mostly to albumin. Newer methods to measure partitioning, using nuclear magnetic resonance (NMR) spectroscopy and fluorescence without separation procedures, have shown that partitioning depends on the concentrations of FFA and albumin, ν, the type of FFA, and the type of phospholipid (38,40,41). NMR experiments monitoring FFA partitioning have shown that pH (41) and temperature (42) also strongly influence the partitioning of FFAs between model membranes and albumin. Decreasing pH from neutral to acid increasingly favors partitioning to membranes, and decreasing temperature favors partitioning to albumin. In the cell, FFAs can bind to FABPs, which have a lower affinity for FFAs than for albumin (43).

UPTAKE AND MEMBRANE TRANSPORT OF FFA

"Uptake" of FFAs is the net disappearance of FFA from plasma or from an in vitro incubation medium into cells, and it involves several distinct steps (Fig. 2): *1*) dissociation of FFA from albumin or a plasma lipoprotein; *2*) adsorption, i.e., diffusion through the diffusion barrier (the space between the cells in the tissue and/or the unstirred water layer adjacent to the plasma membrane) and association to the outer leaflet of the plasma membrane; *3*) movement across the plasma membrane; *4*) dissociation from the inner leaflet to enter the cytosol; *5*) diffusion, either as a monomer or bound to FABP, through the cytosol to sites of binding and utilization (mitochondria, peroxisomes, endoplasmic reticulum); and *6*) metabolism. In most uptake assays, cells are mixed with a medium containing albumin-bound FFA (containing a fraction of ^{14}C-labeled FFA) at variable albumin and FFA concentrations, and the initial $[FFA]_a$ is calculated. Subsequently, the cells are separated from the medium at different time

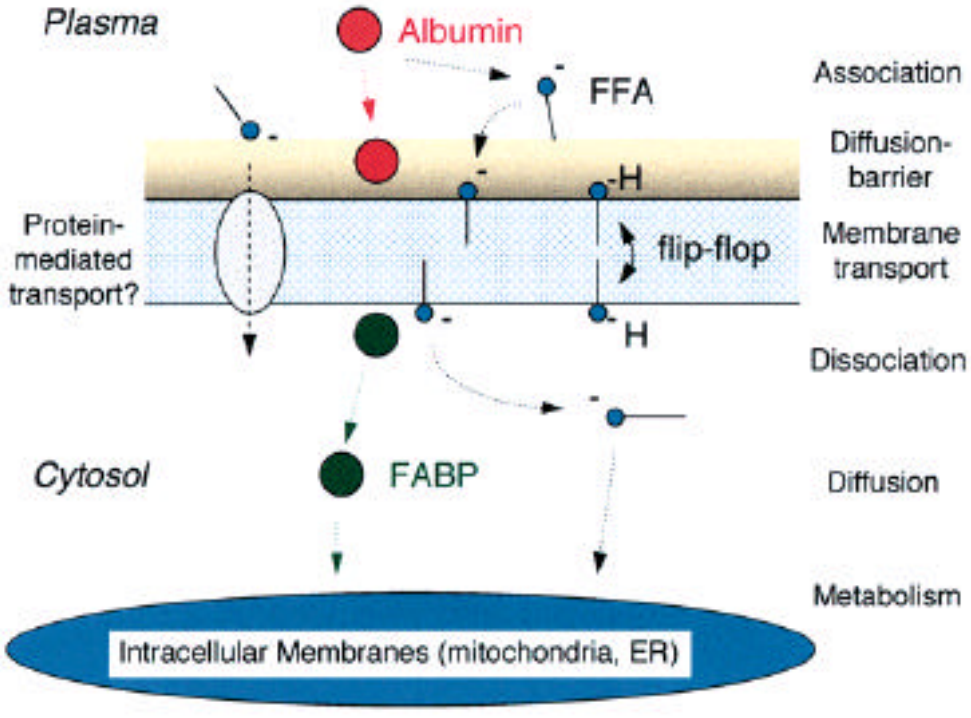

FIG. 2. Key steps of uptake of FFAs into cells. To cross the membrane, FFAs have been hypothesized to diffuse freely through the lipid bilayer or to be transported by a protein. In protein-mediated transport, FFAs could bind either from the external phase or via the membrane lipid phase to a transmembrane protein that transports the anionic form. Free diffusion can occur by flip-flop of FFAs across the plasma membrane in their un-ionized form. For further explanation, see text. ER, endoplasmic reticulum.

intervals, and FFA uptake is evaluated from the amount of radioactive label in the cells. The initial rate of uptake is plotted against the initial unbound FFA for different experimental conditions. In such an assay, one has to ensure whether one measures binding of FFA to the plasma membrane, transmembrane transport, or metabolism. FFAs are esterified within minutes in several tissues such as the brain (44,45), hepatocytes (46), and adipocytes (14,47). In glucose transport studies, transport and metabolism have been distinguished by reconstituting glucose transporters into vesicles that do not metabolize glucose, as described (48,49). In FFA uptake studies, a distinction between binding, transport, and metabolism has sometimes (14,46,50) but not always been taken into account. For our discussions, we define *membrane transport* of FFAs as steps 2–4 outlined above. In our biophysical analysis of membrane transport, we begin with a model membrane consisting of a phospholipid bilayer and no proteins: if any one of the steps of transport in a model membrane is slow compared with rates of metabolism, then passive diffusion may not suffice to supply a cell with FFAs, and more complex transport mechanisms must be considered for transport of FFAs in biological membranes.

ADSORPTION OF FFA TO A PHOSPHOLIPID MODEL MEMBRANE

Initial unbound FFA is not an independent variable. When glucose is added to an aqueous suspension of vesicles, very little will bind to the lipid phase of the plasma membrane, and such binding would be a weak association with the surface rather than incorporation into the hydrocarbon region (15). The aqueous concentration of glucose, which may be in the millimolar range, will be unchanged. In contrast to glucose, FFA will bind avidly to the lipid (membrane) phase. The total amount of FFA that binds to the membrane and the concentration of aqueous FFA after binding equilibrium is reached will depend on many factors, including the form in which the FFA is added (unbound or complexed to albumin).

To illustrate some of the important considerations in carrying out uptake studies with FFAs, we explore the consequences of adding FFAs in different forms and at different concentrations to a system in which they are not metabolized (Fig. 3). In our example, unilamellar phospholipid vesicles (0.2 mmol/l phospholipid) are suspended in an aqueous buffer (pH 7.4) in a cuvette. (This would be the equivalent of ~10^6 cells/ml, assuming that the most important FFA binding moiety is the plasma membrane [with no endogenous FFA] [51], and no metabolism occurs.) The total amount of phospholipid is 500 nmol in 2.5 ml buffer. In the first case (Fig. 3*A*), we add 12.5 nmol of PA as an aliquot of 10 mmol/l K^+ palmitate (the soluble micellar form). The initial concentration of unbound PA is 5 µmol/l, slightly above its solubility limit (Table 1), and the sample must be well mixed to ensure binding to the membrane without precipitation of PA. The final binding equilibrium is quantitated by the very high K_p of ~1.0×10^6. Hence almost all of the PA will bind to the lipid phase. From the K_p we calculate that the final unbound concentration of PA ($[PA]_a$) is now 25 nmol/l, and the concentration in the membrane is 25 mmol/l (based on V_m). Another way to state the concentration of FFAs in the membrane is the mole% with respect to phospholipid, i.e., 2.5 mole% in this example. This is a higher concentration in the membrane than probably occurs in a cell plasma membrane in the typical steady state (52), but it is well below the

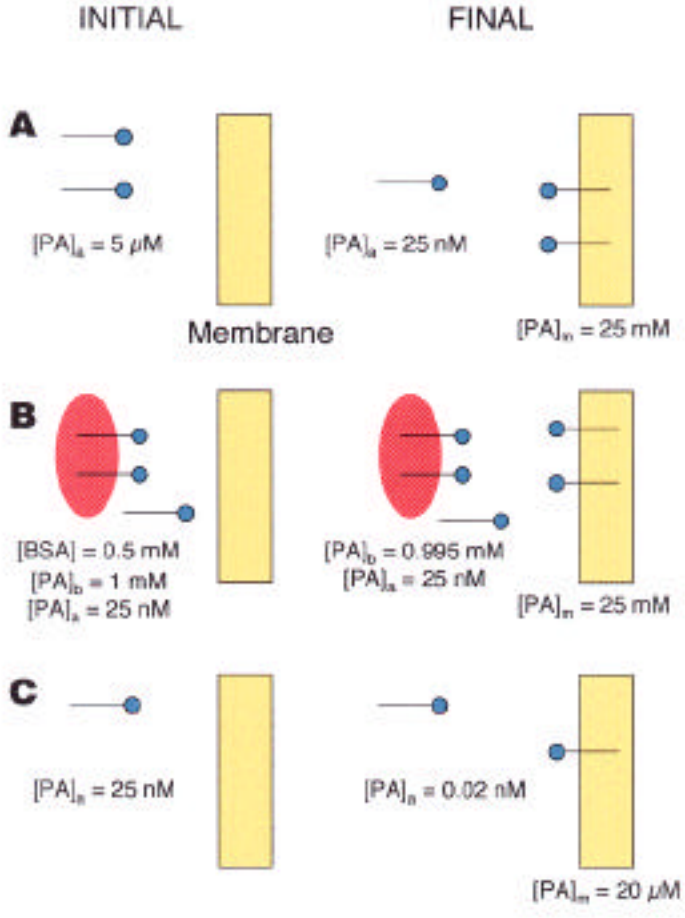

FIG. 3. Initial and final (equilibrium) conditions for PA partitioning to model membranes. The initial and final concentrations of unbound PA ($[PA]_a$), PA bound to albumin ($[PA]_b$), and PA bound to the membrane ($[PA]_m$) were calculated as described in the text. Note that the final concentrations in the membrane are quantitated with respect to the volume of the membrane. *A*: Addition of 5 μmol/l PA as an aliquot of K^+-palmitate without albumin. *B*: Addition of 1 mmol/l PA complexed with 0.5 mmol/l albumin (ν = 2). *C*: Addition of 25 nmol/l PA without albumin. FFA molecules are schematic and not quantitative.

capacity for phospholipid bilayers to adsorb FFAs without disruption of the bilayer structure (53). Because K_p is the ratio of the off-rate constant (k_{off}) to the on-rate constant (k_{on}) for FFA binding, the on-rate constant must be 6×10^6 s^{-1}, based on the measured off-rate constant of 6 s^{-1} (see below), i.e., the adsorption step is very fast and diffusion-controlled (17).

In the second case (Fig. 3*B*), albumin is used as a donor of PA. Now higher amounts of PA can be handled without concern about precipitation of the PA, but the partitioning of PA between albumin, water, and membranes must be considered. Let us add to our model membranes 1.0 mmol/l PA complexed with 0.5 mmol/l albumin, concentrations that could be found in venous blood. With albumin as a donor of PA, and ν = 2, the concentration of unbound PA is maintained at ~25 nmol/l (34). Some of the unbound PA immediately binds to the phospholipid bilayer, and the transfer of PA continues until a new distribution has been reached that is determined by the relative binding affinities of the membrane and albumin for PA. The *rate* of binding of FFAs to the membrane is a function of the k_{on}, the surface area of the membrane, $[FFA]_a$, and the rates by which FFAs leave the binding sites of albumin. New data suggest a half time ($t_{1/2}$) of ~1 s for dissociation of PA or OA (C18:1) from albumin (54), and older data suggest a $t_{1/2}$ of ~10 times longer (40). (Numerous studies have shown that a receptor or a specialized binding protein is not required to transfer FFA from albumin to membranes [40,41,55], although there is still some controversy about FFA transfer from albumin to cells [56].) Thus, in spite of the low aqueous concentrations, the FFA distribution reaches a new equilibrium rapidly (within seconds). The net *amount* of transfer is not limited by the low $[FFA]_a$, and it depends on the total concentration of FFA and on the amounts of phospholipids and albumin. We assume that PA transfers to the membranes until a new equilibrium exists between $[PA]_a$ and PA bound to albumin and the membrane; based on the same K_p as in case 1 (Fig. 3*A*), 12.5 nmol, i.e., only 0.5% of the PA bound to albumin, transfers to the vesicles. The final concentrations of PA in the membrane and water phase are both the same as in case 1, and the amount bound to albumin has not significantly changed. In comparing examples 1 (Fig. 3*A*) and 2 (Fig. 3*B*), we have two cases where the initial concentrations of unbound PA are markedly different, but the final quantity of PA bound to the cells is the same.

In a third example (Fig. 3*C*), we can match the initial aqueous concentration of PA to the last example by adding 0.010 nmol of PA (25 nmol/l) without albumin. In this case, most of the PA will bind to vesicles, but now the final concentration of unbound PA will be much lower than the first two cases. These three examples show that specifying only the concentration of unbound PA is not sufficient to quantify the adsorption of PA to a membrane. In analyzing FFA transport in a multiphase system, the concentration of FFA in the water phase is not an independent variable (13). These complex issues surrounding FFAs are not applicable to glucose.

Implications for transport in cells. The knowledge that there is not a unique relationship between initial unbound FFA and the amount of FFA partitioning into a membrane has implications for studies of FFA transport and uptake in cells. First, if radiolabeled FFAs are used to study FFA transport, the detection of labeled (unesterified) FFAs in the cell fraction does not necessarily tell us about any step except adsorption. The FFAs may have adsorbed to the outer leaflet of the membrane without any translocation through the membrane unless additional experiments showed internalization of FFAs. Second, FFA exchange between membranes and albumin is dynamic, i.e., under conditions where an equilibrium distribution has been reached, FFAs are continuously and rapidly exchanging. Consider an experiment in which albumin with a very low content of bound FFA, including a trace amount of radiolabeled FFA, is added to cells that have some steady-state endogenous concentration of FFA bound to the plasma membrane, as will be the case in general. It is possible to match conditions so that there is no net flux of FFA in either direction, in or out of the cell. The labeled FFA will "report" uptake of FFA, since the labeled FFA will transfer to reach its equilibrium distribution. It is also possible that we have a low enough concentration of albumin and its bound FFA so that there is a *net efflux* of FFA from the cell. Nevertheless, the labeled FFA will still report cellular *influx* of FFA. Hence, uptake assays with labeled FFA (radiolabels or fluorescent labels) may reflect an exchange process rather than an uptake process. Third, quantitation of FFA uptake

based on fractionation procedures may lead to overestimation or underestimation of the FFA adsorbed to a cell. If a very insoluble FFA, such as stearic acid (C18:0), is presented to cells without albumin, some of the FFAs may precipitate but be counted as being taken up into cells if the cells are sedimented along with the precipitated FFAs. Another inaccuracy could be encountered if cells are incubated at room temperature or 37°C with FFA/albumin complexes and cooled to 4°C before separation: partitioning may remove some of the FFA from the cells at the lower temperature (42), resulting in a lower estimate of uptake.

TRANSMEMBRANE MOVEMENT OF FFA IN PHOSPHOLIPID BILAYERS

When FFAs are added to cells in vitro or when high concentrations in the plasma reach cells, *partitioning* into the plasma membrane gives a high concentration of FFA at the outer leaflet, which compensates for the low aqueous FFA and could therefore enhance FFA diffusion fluxes. However, partitioning does not predict whether FFA translocation *through* the membrane will occur spontaneously. A phospholipid molecule such as phosphatidylcholine partitions from the aqueous phase to a preexisting bilayer even more favorably than FFAs but spontaneously crosses the bilayer by flip-flop with a $t_{1/2}$ of days (57), mainly because of the negative charges and the low solubility of the phosphate headgroup in the hydrocarbon region of the phospholipid interior. On the contrary, a diglyceride molecule, which has two carbonyl groups and an OH group but lacks a charged group, partitions very favorably into a phospholipid bilayer and also rapidly flip-flops between the two leaflets (58).

The apparent pK_a of FFA is higher in a membrane than in the water phase. When a long-chain FFA is present at very low concentration in an aqueous compartment at pH 7.4 in an unbound form, the pK_a is ~4.5, and the fully ionized monomeric FFA is the vastly predominant species (28). After the FFA has adsorbed to one leaflet of the phospholipid bilayer, the acyl chain aligns with the acyl chains of the phospholipid, with the carboxyl headgroup positioned at the aqueous interface, and the ionization of the carboxyl group (i.e., its pK_a) is significantly altered. The increase in pK_a on going from the unbound state in the aqueous phase to a membrane phase is a predictable and well-documented property (59–61). The apparent pK_a of the FFA in the environment of the phospholipid (~7.6), measured by ^{13}C-labeled NMR titration experiments, is independent of chain length of saturated FFAs (8–26 carbons) and of unsaturation, as assessed by C18:1, and is not dependent on the relative amount of FFA in the bilayer for the low levels studied (<10 mole% FFA) (59). The pK_a can shift depending on the phospholipid headgroup, the cholesterol content of the phospholipid bilayer, and the ionic strength, but the un-ionized form of the FFA is still abundant (59,60).

Flip-flop of ionized FFA is slow. The transmembrane step of FFA has been the most controversial step of FFA membrane transport, for both theoretical (62,63) and experimental (64,65) reasons. The major theoretical justification for protein-mediated transmembrane transport of FFA has been based on the assumption that the FFA exists solely as an anion at physiological pH (66). Because FFA anions flip-flop very slowly (67), free diffusion would be very slow, and an anion transporter would be needed to overcome the energy barrier of moving the negative charge through the lipid bilayer. Other investigators postulated that the un-ionized FFA could flip-flop much faster without providing proof of such a mechanism (41,67–69). Experimentally, it is difficult to track the movement of FFA within the phospholipid bilayer, in part because of the simplicity of the molecule. Early methods using FFA labeled with a fluorescent group and indirect methods to assess flip-flop were inconclusive. By monitoring the transfer of the labeled FFA from donor vesicles to acceptor vesicles, it was concluded that flip-flop was slow or fast, depending on the type of label (70,71). Experiments that monitored the movement of natural FFA from vesicles to albumin strongly suggested that FFA flip-flop was very fast (40).

Flip-flop causes changes in internal pH. With the understanding of the partitioning and ionization behavior of FFA in membranes, we proposed a method to measure FFA diffusion across the membrane based on the hypothesis that FFAs can move freely and quickly across the bilayer in their un-ionized form and very slowly in their ionized form (19,21,67). To test this flip-flop hypothesis, we developed a fluorescence approach that *1*) allowed a more direct measurement of the transmembrane step of FFA transport than previous methods, *2*) used natural FFA, and *3*) used a fluorescent pH probe (pyranin) that does not interact directly with the FFA. Phospholipid vesicles that were impermeable to protons were prepared with entrapped pyranin. FFA was added into the external buffer as the unbound form or as an albumin complex. According to our hypothesis, FFAs bind to the external leaflet and flip-flop in their un-ionized form. The carboxyl group of the FFA in the inner leaflet reaches ionization equilibrium, producing a measurable decrease in internal pH (pH_{in}). The rate of the pH_{in} decrease in these vesicles reflects the rate of the transmembrane step (Fig. 2), since the adsorption step and ionization processes are very rapid, and the proton is released by the FFA without desorption of the FFA molecule from the inner leaflet. Because of the small internal volume, pyranin can detect the pH change associated with transmembrane movement of FFA.

This simple experiment (Fig. 4) yielded the predicted pH effect after addition of OA. To test our hypothesis further, we added sufficient albumin to bind all the FFA into the external buffer, and the pH_{in} quickly returned to the initial value, as predicted (19,38,67). Additional experiments testing correlates of the flip-flop model have provided evidence in support of the diffusion mechanism (19,38,67). For example, when vesicles are prepared with FFA already present, an increase in the outer pH causes an immediate increase in pH_{in} as a result of net movement of some of the FFA to the outer leaflet to reach the new ionization equilibrium (72). We also generated FFA within the outer leaflet of small unilamellar vesicles (SUVs) by the addition of phospholipase A_2 and observed gradual acidification of the inner volume that stopped when phospholipase A_2 activity was inhibited by EDTA (38).

Flip-flop of un-ionized FFA is very fast. The illustration of OA flip-flop in Fig. 4 shows a time course faster than 1–2 s, the mixing time and the time resolution of the fluorimeter used in our initial studies (19,38). Therefore, the $t_{1/2}$ for the transmembrane step must be <1 s. In subsequent experiments aimed at measuring the actual value with stopped-flow fluorimetry, we found that both the adsorption and transmembrane steps were complete in much less than 1 s. The $t_{1/2}$ was <10 ms when SUVs (d ~25 nm) were used as model membranes and

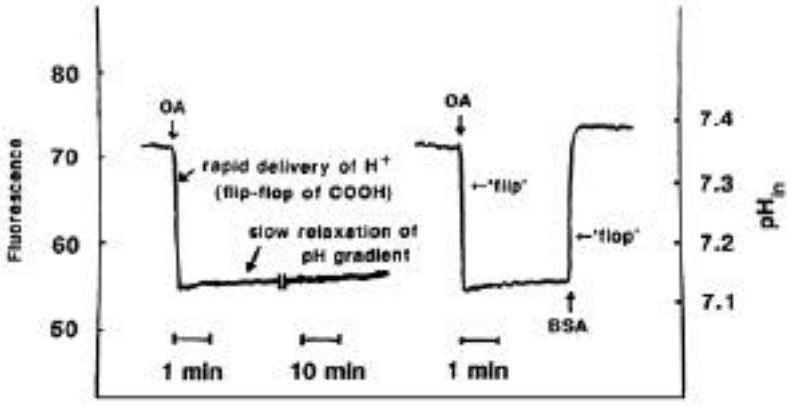

FIG. 4. Evidence for the flip-flop of FFAs in model membranes (phospholipid bilayers). Left trace: to SUVs with a trapped fluorescent pH indicator (pyranin), an aliquot of OA is added, which causes an instantaneous drop in pH_{in} from net transfer of protons as predicted by the model described elsewhere (19). The pH gradient does not dissipate because H^+-leakage is slow, but it can be reversed rapidly by removing the OA with BSA (right trace). Reprinted from Kamp and Hamilton (19).

~25 ms when large unilamellar vesicles (LUVs) (d ~100 nm) were used (73). The presence of up to 20 mole% cholesterol in SUVs did not increase the $t_{1/2}$, and we observed no clear differences in the kinetics of FFA with chain lengths of 12–18 carbons, which with our experimental uncertainty suggested the rates are the same within an order of magnitude. This is consistent with a model of transmembrane diffusion in which the major free energy barrier for translocation is that associated with the transfer of the uncharged carboxyl group into the nonpolar bilayer interior (62,63). In additional experiments (at the lower time resolution) that used FFA/albumin complexes as donors of FFA, we found fast kinetics ($t_{1/2}$ of <1–2 s) of the combined steps of transfer of FFA from albumin and transmembrane diffusion (38).

Other authors have used a similar assay to test the flip-flop rates of a variety of fatty acids and found that most naturally occurring FFAs flip-flop fast, whereas some FFAs, such as 1,12-dodecanedioic, 6-phenylhexanoic, and 12-hydroxylauric acid, appear to flip-flop at much slower rates (74). However, in our own studies we have not yet found any FFA or FFA derivative that displays slow flip-flop; recently, we have shown that oxidized FFAs (13-HODE and 15-HETE, which have polyunsaturation and an OH-group) exhibit flip-flop with a $t_{1/2}$ of <1 s (B. Ek, F. Zhang, J.A.H., unpublished observations). One of the widely used fluorescent labels for FFA is the anthroyloxy group, which may produce experimental artifacts (73) or may slow down the flip-flop by several orders of magnitude compared with natural FFA (65).

Our studies of FFA flip-flop (73) agree with the faster time scales suggested from studies with pyrene-labeled FFA (71) and natural FFA (40). Additional factors in simple model membranes that could influence the flip-flop rate of a specific FFA include vesicle diameter and cholesterol content (65). There is now good agreement about the very fast movement (millisecond time scale) of FFAs across SUVs and LUVs. However, larger vesicles with high cholesterol content are reported to increase the $t_{1/2}$ to 1–3 s (65). It is not clear whether this result is mainly attributable to curvature of the bilayer. However, in considering cells it is important to note that cell membranes are not necessarily uniform in curvature or lipid composition. For example, the plasma membrane of adipocytes contains numerous caveolae, which are enriched in cholesterol and have a high curvature (76). In any case, the model system data with natural FFA now provide consistent and conclusive evidence that a phospholipid bilayer *is not* a barrier to the rapid diffusion of FFAs.

Implications for transport in cells. In spite of limitations of simplified model systems, the lipid bilayer is the fundamental structure of most biological membranes, and we predict that, in contrast to the case of glucose, membrane proteins are not universally required for transmembrane transport of FFA. The argument that FFA cannot diffuse across the lipid bilayer because the molecule is negatively charged is readily countered by knowledge of the ionization properties of FFA in membranes and by our measurement of the rapid flux of the un-ionized form. Movement of the anion is not essential for net flux. Furthermore, because of the partitioning of FFA into the lipid bilayer in membranes, a potential membrane transporter would have to compete with the rapid diffusion of millimolar concentrations of FFA in the plasma membrane even if the concentration of FFA in the cytosol is only in the nanomolar range (77).

Our hypothesized mechanism predicts that other amphipathic molecules might penetrate a lipid membrane provided they can assume an uncharged form in the phospholipid interface. In fact, previous to our fluorescence studies of fatty acids, it had been proposed that unconjugated bile acids diffuse through membrane by flip-flop of the un-ionized form (78,79). NMR data provided direct measurement of fast flip-flop (millisecond time range) of cholic acid and other unconjugated bile acids in SUVs (78). The flip-flop of uncharged amphipathic molecules also has to be considered in studies of multidrug-resistance proteins in bacteria (80).

Another very important consideration about the transmembrane step of FFA transport is that during lipolysis of intracellular triglyceride droplets, adipogenic cells (e.g., adipocytes) release large quantities of FFA rapidly and efficiently. Any mechanism proposed for the transmembrane step must address this issue. The flip-flop mechanism allows rapid movement in either direction, according to the FFA concentration gradient.

DESORPTION OF FFA FROM PHOSPHOLIPID BILAYERS

Desorption is slower than flip-flop and adsorption. Once the FFA has reached the inner leaflet of the plasma membrane, it must move to intracellular sites for utilization, except for the fraction that might be activated (esterified to CoA) in the plasma membrane. Can this occur by spontaneous desorption and diffusion through the cytosol? Now we need to consider the rates by which FFAs desorb spontaneously from a simple (protein-free) phospholipid bilayer. Although several studies have measured the transfer of FFA from vesi-

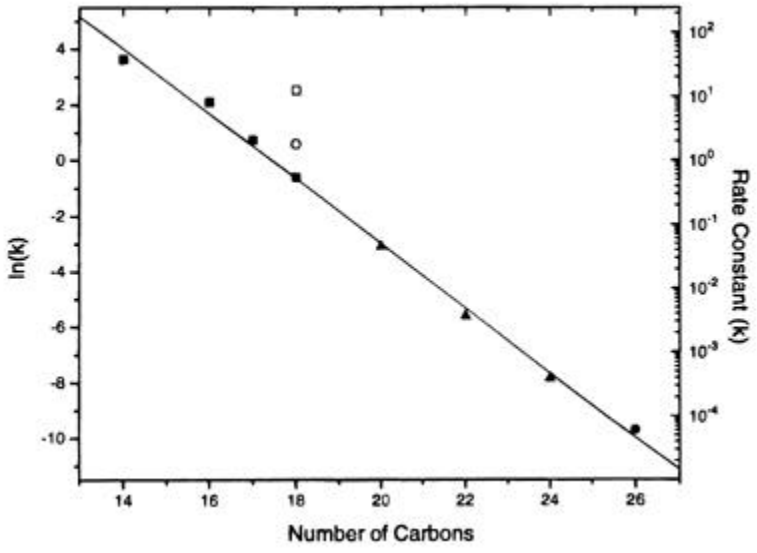

FIG. 5. Dependence of the desorption rate constant (k_{off}) at 24°C on the FFA chain length. Solid symbols represent saturated FFAs with chain lengths of 14–26 carbons. The k_{off} values for C14:0, C16:0, C17:0, C18:0 (■), C18:1 (○), and C18:2 (□) were obtained by transfer studies using stopped-flow fluorimetry. The k_{off} values for C20:0, C22:0, and C24:0 (▲) were obtained by on-line fluorimetry. The k_{off} for C26:0 (●) was measured by ^{13}C-labeled NMR methods (86). Reprinted from Zhang et al. (72).

cles to donors (other vesicles or albumin), the kinetics reflect both the transmembrane and desorption steps. It has been argued by some investigators that the desorption step is the slower step (40,71) and by others, the opposite (64,65,70,81).

After studies of the kinetics of flip-flop, we adapted the pyranin assay to measure the transfer of FFA from vesicles and delineated the desorption step clearly (72,73). To derive pseudo-unimolecular rate constants for desorption of FFA (k_{off}), we measured the transfer kinetics for FFA with chain lengths of 14–24 carbons by stopped-flow (C14:0–C18:0, C18:1; C18:2) and on-line (C20:0–C24:0) fluorescence. The calculated rate constant was always lower than the upper limit for flip-flop of the FFA and therefore could be attributed to the desorption step. The k_{off} showed a predictable relationship with FFA chain length (Fig. 5). There is an ~10-fold increase in k_{off} for addition of two CH_2 groups to the aliphatic chain, and an ~5-fold decrease for each double bond in the 18-carbon chain. The activation energy per CH_2 group (–740 cal) is close to the value derived for the partitioning of long-chain FFAs between an organic phase and water (82). Thus, kinetics of desorption reflect the thermodynamics of partitioning, which is expected, since K_p equals k_{on}/k_{off}, and k_{on} is diffusion controlled and not as strongly dependent on the chain length (17).

There is now general agreement about the kinetics of desorption of FFA from phospholipid vesicles. The earlier transfer data for natural FFA (40) and pyrene-labeled FFA (71) clearly measured the desorption step, as the authors of those studies argued. New data for the desorption of polyunsaturated FFAs show a predictable relationship between the k_{off} and the unsaturation of the acyl chain (54), as suggested by our comparison of stearic acid, OA, and linoleic acid (Fig. 5). The addition of double bonds increases the desorption rate in an additive manner, and the polyunsaturated FFAs with a 20–24 carbon chain have k_{off} values comparable to those of typical dietary FFAs (54). The curvature of the phospholipid vesicle does not seem to greatly influence the desorption rates. The rate constants for specific FFAs reported for LUVs and giant unilamellar vesicles (65) are similar to the rate constants for SUVs (Fig. 5).

Implications for transport in cells. The rates of transfer of FFAs between vesicles and acceptors and the derived rate constants for desorption provide evidence that typical long-chain FFAs can, in principle, desorb spontaneously and rapidly ($t_{1/2}$ of <1 s) from either the outer or inner leaflet of the plasma membrane (72). FFAs can desorb from the outer leaflet of the plasma membrane into the extracellular aqueous phase and bind to albumin. FFAs can leave the inner leaflet and bind to FABP or diffuse as monomers to reach intracellular membranes and sites of utilization (Fig. 2). Although it has been suggested that the intracellular FABP may facilitate desorption of normal dietary FFAs from the plasma membrane (83–85), the fast desorption rates reported for model membranes weakens this hypothesis. Saturated very-long-chain fatty acids (VLCFAs) (e.g., C20:0–C26:0) would seemingly benefit more from such a mechanism, but these FFAs accumulate in cell membranes where they cause damaging effects (86). Long-chain FFAs most likely transfer from the inner leaflet to the cytoplasm and bind to FABP via a diffusion-controlled mechanism. Studies of intracellular FFA transport in heart tissue support this hypothesis (77,87–89). On the other hand, certain types of FABP may release the bound FFAs by collisions or interaction with intracellular membranes (90).

DIFFUSION MODEL APPLIED TO CELLULAR UPTAKE OF FFA

Let us consider the uptake and removal of FFA from a cell exposed to albumin according to predictions of the diffusion mechanism. First, assume that the cell contains no FFA initially and that there is no intracellular metabolism of FFA. The incoming albumin will deliver FFA to the plasma membrane rapidly, potentially within a second or as fast as mixing and diffusion permit. As soon as FFAs reach the outer leaflet of the plasma membrane, some will flip-flop in their unionized form to equalize their concentration across the membrane. Spontaneous desorption of FFA from the inner leaflet to binding sites on intracellular membranes and proteins (mainly FABP) will begin immediately. At some time, possibly within seconds, an equilibrium distribution among the binding sites for FFA (albumin, membranes, and FABP) and a low concentration of unbound FFA, equal on each side of the plasma membrane, will be reached. The vast majority of FFA in a cell is probably bound to the plasma membrane, based on calculations of typical total FFA amounts in cells and

the abundance and affinities of the different binding sites (77,91). The almost millimolar concentrations of FFA bound to albumin could lead to millimolar concentrations of FFA in the plasma membrane (Fig. 3*B*). The concentration of unbound FFA probably remains in the nanomolar range on both sides of the plasma membrane.

Intracellular utilization of FFAs (except for small amounts of polyunsaturated FFA that are oxidized directly) begins with the enzymatic conversion of FFAs to acyl-CoAs (which are subsequently stored mainly in acylated lipids or oxidized in the mitochondria) (2). Because the activation of long-chain FFAs takes place in membranes, the concentration of FFAs in the membranes, and not the aqueous concentration, is the appropriate concentration to consider for intracellular metabolism (13,91–93). As soon as acyl-CoA is formed, the FFA is trapped in the cell, since the flip-flop of acyl-CoA is very slow or nonexistent (94,95), and a concentration gradient of FFA across the plasma membrane will be generated. The FFA can be replaced by the external pool of albumin-bound FFA, at a rate depending on the concentration of albumin and the bound FFA, the rate of desorption of FFA from albumin, and the concentration of external unbound FFA. Most available data indicate that these kinetics are faster than the kinetics of intracellular metabolism, i.e., that the desorption of FFA from albumin does not limit metabolism (51,55,91).

Intracellular metabolism of FFAs can also consist of lipolysis of triglyceride stores to generate FFAs, for either oxidation within the same cell or export to other cells. The increase in intracellular FFAs will increase the concentration of FFAs in the plasma membrane, and FFAs will quickly leave the cell to bind to albumin. The exit of FFAs will continue until a new equilibrium distribution is reached. When the albumin attains a high content of FFAs, the partitioning relationships will force FFAs to accumulate in the cell until lipolysis stops. If the albumin that is loaded with FFAs is replaced by albumin with a low content of FFAs, as can occur in the circulation, the efflux of FFAs can continue.

Finally, an important prediction of the diffusion mechanism is that all FFAs (different chain lengths, degrees of unsaturation, and extent of oxidation) can gain entry to a cell and can leave it. Cells cannot exclude entry of a specific FFA at the plasma membrane. This means that fluctuations in extracellular aqueous FFAs in the blood, which are uniquely related to the fluctuations in FFA/albumin molar ratios (varying from 0.5 to 6) lead to similar fluctuations of the intracellular FFA concentrations. Such wide fluctuations need to be considered in studies of the signaling functioning of FFA and its relation to the causes of diabetes.

PROTEIN-MEDIATED MEMBRANE TRANSPORT OF FFA

The information from the model systems can be used to conclude that a protein is not obligatory for the fundamental steps of transport of FFA in lipid bilayers: adsorption, transmembrane movement, and desorption. Involvement of proteins in the overall process of FFA uptake in cells (Fig. 2) has not been and cannot be excluded, as discussed below. Earlier studies supporting diffusion of FFA into cells may not have been convincing to many investigators because much of the data was based on medium- and short-chain FFAs and because of the complications (experimental and interpretive) with long-chain FFAs. At the same time, several investigators interpreted data for cellular uptake of FFA as requiring protein involvement or an undefined biological mechanism (20), and some investigators have identified putative transport proteins. We first review the types of evidence cited in support of FFA transporters and then discuss specifically some of the putative FFA transport proteins.

Saturation effects. Data for FFA uptake into cells have often been analyzed like data for glucose transport (Fig. 1*A*). One interpretation of this curve is that it represents both a saturable and a nonsaturable component, as shown in many studies (Fig. 1*B*). However, what is the true substrate concentration? Is it the total amount of FFA complexed with albumin, the amount of FFA adsorbed to the lipid bilayer in the membrane, or the amount of initial unbound FFA? As discussed above, the concentrations of unbound long-chain FFAs are too low to measure directly, and estimates of equilibrium binding constants for FFA/albumin complexes obtained by classical partitioning measurements (31) and by ADIFAB fluorescence (34) have differed significantly. Figure 1*B* uses the more recent estimates from ADIFAB. Data from other published studies have used the higher estimates derived from the former studies and should be replotted with newer estimates of $[FFA]_a$ to determine whether saturation effects are still seen.

Alternative approaches for quantitative analysis of steady-state FFA uptake based on properties of lipids and using data from model systems have been formulated (13,40,51, 91–93). Uptake of FFA in perfused livers could be explained completely by assuming that FFAs enter the cell by free diffusion and that the rate of metabolism is proportional to the amount of FFA that partitions into the plasma membrane (13). Saturation of the rate of steady-state uptake of FFA by cells was attributed to the physical chemical properties of the system (partitioning) and not to a transporter. As shown in Fig. 6, when cells are exposed to increasing concentrations

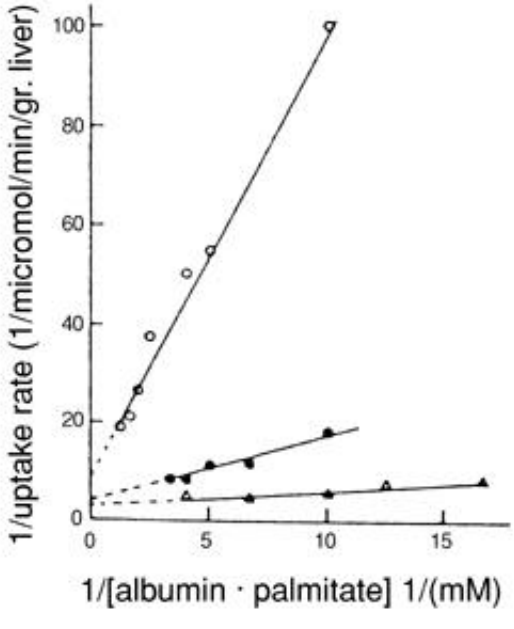

FIG. 6. The rate of uptake of palmitate by perfused liver in the steady-state as a function of concentration of PA/albumin complexes at three mole ratios. Data are plotted in the double reciprocal form. Each line corresponds to a different constant ratio (ν) of moles FFA/moles albumin: ν = 0.5 (○), 2.0 (●), or 4 (▲). Reprinted from Noy et al. (51).

of PA in complex with albumin at a fixed ratio (ν), Michaelis-Menton kinetics are observed for each ν, but the K_m and V_{max} depend on the ν. The uptake saturates at higher concentrations of the albumin/PA complex, because albumin limits the binding of PA to the membrane in a manner depending on ν (Fig. 3). There is no unique K_m and V_{max} as in the case of glucose (Fig. 1*A*). This analysis, although often overlooked, emphasizes fundamental differences between the membrane transport of water-soluble and lipid-soluble molecules.

The time course of FFA uptake may also show a nonsaturable and a saturable component for reasons other than saturation of a transport protein or partitioning. For instance, in analysis of their own data on heart myocytes, DeGrella and Light (96,97) argued that the nonsaturable component represented accumulation of FFAs and the saturable component represented the portion of FFAs converted to metabolic products rather than a transporter.

Competition. In some classical uptake assays, the initial rate of uptake of one kind of FFA can be decreased by another kind of FFA; this has been taken as evidence for protein-mediated FFA membrane transport (10). With the high concentrations of FFA used in competition studies (50,98), FFA uptake as measured by radiolabeled FFAs is expected to decrease because of partitioning; thus, decreases in the binding of the same or another long-chain FFA are not proof of protein-mediated uptake. "Control" experiments with medium-chain FFAs (octanoic acid) or glucose (98) are inconclusive because these molecules partition weakly (octanoic acid) or almost not at all (glucose) into the lipid bilayer. Furthermore, in competition studies with cells, using conditions with low FFA/albumin mole ratios, very little FFA may be transferred from albumin to the plasma membrane, and the uptake of a specific FFA could be controlled mainly by metabolism. Although protein-mediated transport may appear to be an attractive mechanism because of its potential selectivity of transport of FFA, we believe that discrimination of FFA occurs mainly at the enzymatic steps of FFA utilization.

Inhibitors. The observation that FFA uptake into certain cells can be decreased by adding inhibitors has frequently been taken as evidence for protein mediation of FFA uptake. In studies of FFA uptake into heart myocytes, the complex effects of numerous inhibitors and reagents on FFA accumulation and formation of the specific metabolic products of FFA could all be interpreted as effects on one or more of the metabolic transformations of FFA (96,97). An important precaution when adding chemical reagents is that some agents thought to be impermeable to the plasma membrane may actually enter the cell and inhibit intracellular processes or bind to internalized fluorescent probe molecules. In recent compilations of inhibitors (10), it is interesting to note that many are known metabolic inhibitors, including phloretin, an inhibitor of glucose transport, and some others are inhibitors of known anion channel proteins.

The presence of inhibitors in experiments with FFA uptake generally decreases the maximal uptake of FFAs. The extent of decrease has been attributed to the contribution of protein-mediated transport, and the remaining transport has been attributed to diffusion. In most cases, the diffusion component amounts to the major component of the overall uptake. However, the inhibitors generally do not remove the "saturation" effect to reveal only the linear relationship suggested by some to represent a diffusion process (Fig. 1*B*). Metabolic inhibitors could decrease both the rate and maximal uptake of FFAs by affecting metabolic steps of uptake. Nonmetabolic inhibitors could exert indirect effects, such as an effect on partitioning (13). If the concentration of inhibitors is high and the FFA:albumin ratio is low, these imposed conditions could greatly decrease the partitioning of FFAs into the membrane.

Expression. Some of the newer putative FFA transport proteins have been discovered by overexpression of the protein into cells. The hypothesis is that if increased FFA uptake is observed, the expressed protein is a transporter of FFAs. Expression of putative FFA transporters has generally resulted in increases of 2- to 10-fold in the amount of FFA uptake over the control rather than the much larger increases that would be expected if the transport of FFAs was analogous to glucose (Fig. 1). In some cases, there has been no increase at all (99). As discussed below, we believe that experiments expressing or deleting a specific protein implicated in FFA transport cannot be conclusive as to the function of the protein.

PUTATIVE MEMBRANE TRANSPORTERS FOR FFA

Fatty acid transporter. A large molecular weight (88 kDa) glycosylated protein was described as an FFA transporter (designated fatty acid transporter [FAT]) in the plasma membrane of adipocytes. The protein could be labeled covalently by reactive esters of long-chain FFAs (100). Other investigators showed that a very similar protein (and with sequence homology to the CD36 protein) was covalently labeled by FFAs and questioned whether acylation of the protein could explain enhanced uptake of FFA (101). Later studies by the original investigator found that FAT is identical to CD36 (10), a known lipoprotein receptor. Adipocytes isolated from a CD36 null mutation showed small (less than twofold) or no differences in the uptake of FFA compared with the wild-type, depending on the FFA:albumin ratio used in the assay (102). Expression of the protein in fibroblasts showed the characteristic saturation of FFA uptake and inhibition by phloretin (103). However, expression of FAT in a heart muscle cell line showed no effect on FFA uptake (99). Does this mean that in some cases, FAT is not active in transport of FFA because another protein that participates with FAT in FFA transport is not present, or that FAT is not a transporter of FFA?

Fatty acid binding plasma membrane protein. The 40-kDa fatty acid binding plasma membrane protein (FABPpm) is associated with the plasma membrane but is not a transmembrane protein (104,105). Originally found in the liver, antibodies raised against this protein revealed a wide distribution in tissues (10). Its function as an FFA transporter was based on the observation of saturation kinetics and inhibition by antibodies directed against the protein (9,105). The studies were complicated by the fact that FABPpm is identical to mitochondrial glutamic-oxaloacetic transaminase, an enzyme involved in amino acid synthesis (105–107).

Fatty acid transport protein. Fatty acid transport protein (FATP) was discovered by an expression cloning strategy (108). The authors argued that the function of previous putative FFA transporters had not been proved, but the overexpression experiments proved the function of FATP. However, there are limitations to the interpretations of overexpression experiments in general, as noted for FAT. Furthermore, FATP has an AMP binding site and an amino acid sequence similar

to that of very-long-chain acyl-CoA synthetase, raising the possibility that FATP is actually an enzyme (10).

Fatty acid transporter 1. The gene for fatty acid transporter 1 (FAT1) in yeast was first identified by its homology to the gene for FATP (109). Deletion of the gene was associated with decreased uptake of long-chain and fluorescent FFAs into cells and had no effect on long-chain acyl-CoA synthetase activity. Other investigators later reported that FAT1 deletion mutants had markedly decreased very-long-chain acyl-CoA synthetase activity and accumulated VLCFAs, whereas uptake and metabolism of long-chain FFAs were not affected (110). Very-long-chain acyl-CoA synthetase activity was demonstrated in subcellular fractions containing microsomes and peroxisomes. Because the earlier investigators did not assay activity toward VLCFAs, they may have incorrectly described the function of this protein.

Uncoupling proteins. The only putative FFA transporters that have been purified and reconstituted into model membranes are the uncoupling proteins (UCP1, UCP2, and UCP3) found in the inner mitochondrial membrane of various tissues (111–116). UCP-mediated uncoupling is activated by FFA, and the role of the protein was thought to be in direct transport of H^+ or transport of FFA molecules (68,111,112,114,115,117). To clarify its precise role and regulation, UCP1 was reconstituted into LUVs comprised of phospholipids and containing a pH-sensitive fluorophore in the internal aqueous volume (112,114,118,119). The model for the function of this protein (112,114) is consistent with the physical properties of FFAs discussed above: FFAs diffuse across the membrane in their uncharged form, inducing H^+ transport, and UCP acts as a pure anion transporter to allow FFAs to act as cycling protonophores. Binding of the anionic form of the FFA to UCP is consistent with known binding modes of other fatty acid binding proteins, namely, albumin and FABP, which bind the anionic form and not the protonated form of the FFA (41,43).

NEW EVIDENCE FOR THE DIFFUSION MECHANISM IN CELLS

Given the caveats of most experimental approaches to discern the mechanism(s) of FFA transport across membranes, it is important to develop new approaches to study FFA entry into, and exit from, cells. The diffusion mechanism, as discussed in detail above, predicts that *1*) pH changes will accompany FFA flip-flop and *2*) pH gradients will affect the transmembrane distribution of FFAs and their flux (19). The predictions have been validated in model membranes and can be tested in cells. Whereas many studies of FFA transport do not (or cannot) distinguish FFA adsorption to the outside of the cell (or the outer leaflet of the plasma membrane) from movement across the plasma membrane, pH changes in the cytosol of a cell monitor arrival of the FFA at the inner leaflet of the plasma membrane (21). The predictions of the flip-flop model have provided new evidence for the diffusion mechanism of long-chain FFAs, using methods similar to those previously applied to short-chain FFAs (21).

We first investigated the movement of FFAs into pancreatic β-cells loaded with a pH fluorophore. FFAs presented to the cell, either unbound or bound to albumin, caused a pH decrease in the cytoplasm that was reversed by adding excess albumin to the external buffer (120). The pH changes were in accord with the predictions of the flip-flop hypothesis and partitioning. The time course of the pH change ($t_{1/2}$ of ~1 min) was slow compared with simple phospholipid bilayers. However, the experiment measures the rate of binding to the outer leaflet of the plasma membrane as well as the rate of transmembrane movement of the molecule, and in the β-cell transfer of FFAs through the unstirred water layer and the glycocalyx could be slower than flip-flop. Nevertheless, the results showed that FFAs diffuse in and out of β-cells within a minute.

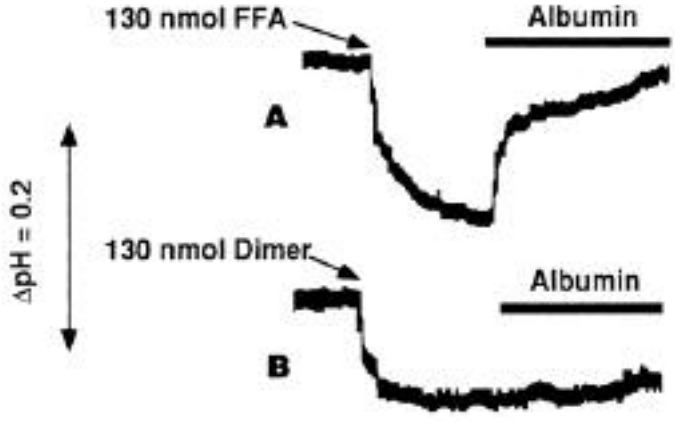

FIG. 7. The time course of change in pH_{in} after addition of FFAs and analogs to suspensions of rat adipocytes. Cells (0.6 mg protein/ml) were suspended in 1.3 ml Krebs buffer without albumin at pH 7.4 and 30°C. *A*: The acidification caused by the addition of 130 nmol OA. Albumin caused a rapid partial recovery of pH_{in}, reflecting rapid removal of FFAs from the cells. *B*: Addition of 130 nmol FFA dimer causes prolonged acidification that is not reversed by albumin. These data demonstrate the flip-flop mechanism in the plasma membrane of adipocytes. Reprinted from Civelek et al. (47).

Another important cell to investigate with new methods is the adipocyte, which has two putative FFA membrane transport proteins (FAT and FATP). Changes in intracellular pH in adipocytes consistent with passive diffusion by flip-flop were observed (47). We found a dose dependency of the pH change, and in some experiments, the pH decrease exhibited a fast component ($t_{1/2}$ of <1 min) followed by a slower component (Fig. 7*A*). (In recent studies with lower amounts of added fatty acids, we have observed a decrease within seconds of the pH_{in} of adipocytes without a slower component [F.K., B.E. Corkey, J.A.H., unpublished observations].) Addition of albumin to the buffer rapidly reversed most of the pH decrease, showing that FFAs can rapidly leave the cell by free diffusion and bind to albumin. In the same study, FFA was detected in the external medium immediately after stimulation of intracellular lipolysis.

One might argue that cotransport of an FFA anion and a proton by a protein would give the same pH changes as flip-flop of the un-ionized FFA. To test the diffusion hypothesis more rigorously, we investigated other amphipathic molecules that would be very unlikely to bind to, or be transported by, a transporter of FFA. First, we used a cross-linked dimer of OA, a double-chain amphiphile that in model systems exhibited rapid flip-flop and did not bind to albumin (120). Addition of this molecule to adipocytes produced a pH decrease that was not reversed by albumin (Fig. 7*B*). The pH increased very slowly after the addition of the dimer, which is slowly metabolized, suggesting that the more rapid return to equilibrium pH with OA reflects its metabolism (Fig. 7*A*).

A basic amphiphile, tetradecylamine, produced a pH increase (47), which arises from the equilibration of uncharged and positively charged molecules in the lipid interface and rapid movement of the uncharged form. Taken together, the results are consistent with the flip-flop mechanism and inconsistent with a protein transporter mechanism.

Other investigators examined the transport of FFAs into adipocytes under conditions where the intracellular and extracellular pH were varied (121). The results were also consistent with the flip-flop hypothesis (19) and its predictions of the effects of pH gradients. An important aspect of this study (121) was that it used conventional radioisotope uptake assays and exposed cells to a lower FFA:albumin ratio ($\nu = 0.5$) that reflects basal conditions. Although it is sometimes argued that *only* low FFA:albumin ratios are physiologically relevant, adipocytes especially may be exposed to high FFA:albumin ratios during lipolysis of triglycerides in adjacent endothelial cells.

Several other studies have provided evidence for passive diffusion of FFAs on the basis of pH effects. OA caused intracellular acidification in isolated peripheral blood neutrophils, and increasing the external pH reduced the magnitude of the pH_{in} decrease (122), as predicted (19,38). FFA transport across the plasma membrane of hepatocytes was also affected in a manner predicted by the flip-flop mechanism when pH gradients were manipulated (123).

Another new approach for studying FFA transport across membranes is entrapment of the fluorescent-labeled FABP ADIFAB, or albumin, which has a natural fluorescence. When FFA is added to phospholipid vesicles with one of these proteins entrapped in the inner volume, the combination of the three fundamental steps of transport (adsorption, transmembrane movement, and desorption) is reported. In an application to red blood cell ghosts, analysis of fluorescence data provided by both ADIFAB and pyranin led to the conclusion that passive diffusion can account for FFA transport across ghosts, even at low FFA concentrations (124).

FFA transport studies in the erythrocyte illustrate the controversies surrounding FFA transport. Studies previous to those discussed above also had led to conflicting conclusions. Transport of OA across erythrocyte ghosts to entrapped albumin was found to be rapid ($t_{1/2}$ of <15 s) and postulated to occur by a "simple diffusive flip-flop process" (125). A much different conclusion, that *specific* protein transporters are present for *specific* FFAs, was reached in another analysis of the kinetics of movement of several FFAs across ghosts to reach entrapped albumin (126). In this view, different proteins act as gatekeepers to allow specific FFAs to move across the membrane. However, the membrane is considered impermeable to FFAs, which we argue is not a defensible hypothesis. Fluorescent-labeled proteins have potential applications for studies of FFA transport in cells with a cytosol. Although they cannot readily be introduced into cells, they can be microinjected into the cytoplasm. However, these may not be ideal probes because in the complex environment within the cell there may be other ligands that interfere with measurements of FFA concentrations.

DISCUSSION

Because of their low aqueous and high lipid solubility, FFA transport studies must be approached differently than glucose transport studies. FFAs partition highly into the lipid domains of biological membranes, and this property is independent of the model of FFA transmembrane transport, whether protein-mediated or free diffusion. When FFAs are delivered to cells or vesicles as an aliquot of a concentrated solution, the potential for precipitation of the FFA has to be considered. When FFAs are delivered complexed to BSA, the $[FFA]_a$ is in the nanomolar range, as in physiological conditions, but the $[FFA]_a$ is not an independent variable and the amount of FFA that transfers depends on partitioning. Moreover, with albumin present, the uptake of FFAs might be determined by FFA metabolism. These complications have to be taken into account in FFA uptake assays. Evidence for FFA transport proteins, such as saturability of uptake, competition between different FFAs, and the effect of inhibitors, is therefore not conclusive. Experiments monitoring intracellular pH, following both protocols for adding FFAs, have demonstrated that the pH_{in} drops instantaneously in vesicles and cells, reflecting flow of FFAs across the membrane. These observations favor the diffusion model involving flip-flop of un-ionized FFAs, although additional testing of the diffusion hypothesis in cells must be done.

The fact that the half times for association, flip-flop, and dissociation processes of FFA in model membranes are all less than a second, whereas metabolic processes usually occur with half times of minutes, suggests that there is no a priori necessity for transport proteins. Moreover, because of the high partition coefficient, steady-state FFA concentrations in the lipid phase of biological membranes are in the millimolar range, while they are probably in the nanomolar range in the cytosolic and extracellular water phases adjacent to the membrane (89); this means that putative membrane transporters would have to compete with fast diffusion fluxes. It seems unlikely that a protein will enhance the transmembrane movement of FFAs. The precise role of plasma membrane proteins in enhancing FFA uptake remains to be defined. We believe indirect roles should be considered. As suggested previously (127), proteins could bind FFAs and thereby increase partitioning into the membrane or could sequester FFAs to a membrane-bound enzyme, thereby enhancing metabolism of FFAs.

ACKNOWLEDGMENTS

We thank Paul Pilch, Henry Pownall, and Marc Prentki for careful reading of the manuscript and helpful comments. We also thank Keith Tornheim, Rick Corkey, and Alex Leaf for many valuable discussions.

REFERENCES

1. DECODE Study Group on behalf of the European Diabetes Epidemiology Study Group: Will new diagnostic criteria for diabetes mellitus change phenotype of patients with diabetes? Reanalysis of European epidemiological data. *BMJ* 317:371–375, 1998
2. Prentki M, Corkey BE: Are the β-cell signaling molecules malonyl-CoA and cytosolic long-chain acyl-CoA implicated in multiple tissue effects of obesity and NIDDM? *Diabetes* 45:273–283, 1996
3. Sleeman MW, Donegan NP, Heller Harrison R, Lane WS, Czech MP: Association of acyl-CoA synthetase-1 with GLUT4-containing vesicles. *J Biol Chem* 273:3132–3135, 1998
4. Cistola DP, Small DM: Fatty acid distribution in systems modeling the normal and diabetic human circulation. *J Clin Invest* 87:1431–1441, 1991
5. Spector AA, Hoak JC: Fatty acids, platelets, and microcirculatory obstruction. *Science* 190:490–491, 1975
6. Bergman RN: New concepts in extracellular signaling for insulin action: the single gateway hypothesis. *Recent Prog Horm Res* 52:359–385, 1997 (discussion 385–387)

7. Boden G: Role of fatty acids in the pathogenesis of insulin resistance and NIDDM. *Diabetes* 46:3–10, 1997
8. Randle PJ, Garland PB, Hales N, Newsholme EA: The glucose–fatty acid cycle: its role in insulin sensitivity and the metabolic disturbances of diabetes mellitus. *Lancet* i:785–789, 1963
9. Stremmel W, Strohmeyer G, Berk PD: Hepatocellular uptake of oleate is energy dependent, sodium linked, and inhibited by an antibody to a hepatocyte plasma membrane fatty acid binding protein. *Proc Natl Acad Sci U S A* 83:3584–3588, 1986
10. Abumrad N, Harmon C, Ibrahimi A: Membrane transport of long-chain fatty acids: evidence for a facilitated process. *J Lipid Res* 39:2309–2318, 1998
11. Glatz JFC, van der Vusse GJ: Cellular fatty acid binding proteins: their function and physiological significance. *Prog Lipid Res* 35:243–282, 1996
12. Glatz JFC, van Nieuwenhoven FA, Luiken JJFP, Schaap FG, van der Vusse GJ: Role of membrane-associated and cytoplasmic fatty acid binding proteins in cellular fatty acid metabolism. *Prostaglandins Leukot Essent Fatty Acids* 57:373–378, 1997
13. Zakim D: Fatty acids enter cells by simple diffusion. *Proc Soc Exp Biol Med* 212:5–14, 1996
14. Berk PD, Zhou SL, Kiang CL, Stump D, Bradbury M, Isola LM: Uptake of long-chain free fatty acids is selectively upregulated in adipocytes of Zucker rats with genetic obesity and non-insulin-dependent diabetes mellitus. *J Biol Chem* 272:8830–8835, 1997
15. Bummer PM, Zografi G: The association of D-glucose with unilamellar phospholipid vesicles. *Biophys Chem* 30:173–183, 1988
16. Spector A: Plasma albumin, a lipoprotein. In *Biochemistry and Biology of Plasma Lipoproteins*. New York, Dekker, 1986, p. 247–279
17. Dietschy J: General principles governing movement of lipids across biological membranes. In *Disturbances in Lipid and Lipoprotein Metabolism*. Dietschy JM, Gotto AM Jr, Ontko JA, Eds. Bethesda, MD, American Physiological Society, 1978, p. 1–29
18. Walter A, Gutknecht J: Monocarboxylic acid permeation through lipid bilayer membranes. *J Membr Biol* 77:255–264, 1984
19. Kamp F, Hamilton JA: pH gradients across phospholipid membranes caused by fast flip-flop of fatty acids. *Proc Natl Acad Sci U S A* 85:11367–11370, 1992
20. Veerkamp JH: Fatty acid transport and fatty acid binding proteins. *Proc Nutr Soc* 54:23–37, 1995
21. Chu S, Montrose MH: An Na^+-independent short-chain fatty acid transporter contributes to intracellular pH regulation in murine colonocytes. *J Gen Physiol* 105:569–615, 1995
22. Hirsch D, Stahl A, Lodish HF: A family of fatty acid transporters conserved from mycobacterium to man. *Proc Natl Acad Sci U S A* 95:8625–8629, 1998
23. Fraser H, Coles SM, Woodford JK, Frolov AA, Murphy EJ, Schroeder F, Bernlohr DA, Grund V: Fatty acid uptake in diabetic rat adipocytes. *Mol Cell Biochem* 167:51–60, 1997
24. Hui TY, Frohnert BI, Smith AJ, Schaffer JE, Bernlohr DA: Characterization of the murine fatty acid transport protein gene and its insulin response sequence. *J Biol Chem* 273:27420–27429, 1998
25. Small DM: Physical properties of fatty acids and their extracellular and intracellular distribution. In *Polyunsaturated Fatty Acids in Human Nutrition*. Bacco U, Deckelbaum RJ, Eds. New York, Raven, 1992, p. 25–40
26. Cistola DP, Atkinson D, Hamilton JA, Small DM: Phase behavior and bilayer properties of fatty acids: hydrated 1:1 acid soaps. *Biochemistry* 25:2804–2812, 1986
27. Hicks M, Gebecki JM: Microscopic studies of fatty acid vesicles. *Chem Phys Lipids* 20:143–252, 1977
28. Ananthapadmanabhan KP, Somasundaran P: Acid-soap formation in aqueous oleate solutions. *J Colloid Interface Sci* 122:104–109, 1988
29. Bell GH: Solubilities of normal alipathic acids, alcohols, and alkanes in water. In *Chemistry and Physics of Lipids*. Amsterdam, North-Holland, 1973, p. 1–10
30. Vorum H, Brodersen R, Kragh-Hansen U, Pedersen AO: Solubility of long-chain fatty acids in phosphate buffer at pH 7.4. *Biochim Biophys Acta* 1126:135–142, 1992
31. Spector AA, Fletcher JE, Ashbrook JD: Analysis of long-chain free fatty acid binding to bovine serum albumin by determination of stepwise equilibrium constants. *Biochemistry* 10:3229–3232, 1971
32. Richieri GV, Ogata RT, Kleinfeld AM: A fluorescent-labeled intestinal fatty acid binding protein. *J Biol Chem* 267:23495–23501, 1992
33. Richieri GV, Ogata RT, Kleinfeld AM: Thermodynamics of fatty acid binding to fatty acid binding proteins and fatty acid partition between water and membranes measured using the fluorescent probe ADIFAB. *J Biol Chem* 270: 15076–15084, 1995
34. Richieri GV, Anel A, Kleinfeld A: Interactions of long-chain fatty acid and albumin: determination of free fatty acid levels using the fluorescent probe ADIFAB. *Biochemistry* 32:7574–7579, 1993
35. Richieri GV, Kleinfeld AV: Unbound free fatty acid levels in human serum. *J Lipid Res* 36:229–239, 1995
36. Huang C, Mason JT: Geometric packing constraints in egg phosphatidylcholine vesicles. *Proc Natl Acad Sci U S A* 75:308–310, 1978
37. Langner M, Isac T, Hui SW: Interaction of free fatty acids with phospholipid bilayers. *Biochim Biophys Acta* 1236:73–80, 1995
38. Kamp F, Hamilton JA, Westerhoff HV: Movement of fatty acids, fatty acid analogues, and bile acids across phospholipid bilayers. *Biochemistry* 32:11074–11086, 1993
39. Anel A, Richieri GV, Kleinfeld AM: Membrane partition of fatty acids and inhibition of T-cell function. *Biochemistry* 32:530–536, 1993
40. Daniels C, Noy N, Zakim D: Rates of hydration of fatty acid bound to unilamellar vesicles of phosphatidylcholine or to albumin. *Biochemistry* 24: 3286–3292, 1985
41. Hamilton JA, Cistola DP: Transfer of oleic acid between albumin and phospholipid. *Proc Natl Acad Sci U S A* 83:82–86, 1986
42. Ho JK, Hamilton JA: Temperature-dependent partitioning of fatty acids between albumin and model membranes (Abstract). *Biophys J* 72:A-313, 1997
43. Cistola DP, Walsh MT, Corey RP, Hamilton JA, Brecher P: Interactions of oleic acid with liver fatty acid binding protein: a carbon-13 NMR study. *Biochemistry* 27:711–717, 1988 (Erratum published in *Biochemistry* 28:3628, 1989)
44. Robinson PJ, Noronha J, DeGeorge JJ, Freed LM, Nariai T, Rapoport SI: A quantitative method for measuring regional in vivo fatty acid incorporation into and turnover within brain phospholipids: review and critical analysis. *Brain Res Brain Res Rev* 17:187–214, 1992
45. Washizaki K, Smith QR, Rapoport SI, Purdon AD: Brain arachidonic acid incorporation and precursor pool specific activity during intravenous infusion of unesterified [^{3}H]arachidonate in the anesthetized rat. *J Neurochem* 63:727–736, 1994
46. Stremmel W, Berk PD: Hepatocellular influx of [^{14}C]oleate reflects membrane transport rather than intracellular metabolism or binding. *Proc Natl Acad Sci U S A* 83:3086–3090, 1986
47. Civelek VN, Hamilton JA, Tornheim K, Kelly KL, Corkey BE: Intracellular pH in adipocytes: effects of free fatty acid diffusion across the plasma membrane, lipolytic agonists, and insulin. *Proc Natl Acad Sci U S A* 93:10139–10144, 1996
48. Schürmann A, Monden I, Joost HG, Keller K: Subcellular distribution and activity of glucose transporter isoforms GLUT1 and GLUT4 transiently expressed in COS-7 cells. *Biochim Biophys Acta* 1131:245–252, 1992
49. Xiao L, Xie WS, Yang FY: A method for unidirectional reconstitution of human erythrocyte glucose transporter. *Biochem Mol Biol Int* 44:1217–1223, 1998
50. Bonen A, Luiken JJ, Liu S, Dyck DJ, Kiens B, Kristiansen S, Turcotte LP, Van Der Vusse GJ, Glatz JF: Palmitate transport and fatty acid transporters in red and white muscles. *Am J Physiol* 275:E471–E478, 1998
51. Noy N, Donnelly TM, Zakim D: Physical-chemical model for the entry of water-insoluble compounds into cells: studies of fatty acid uptake by the liver. *Biochemistry* 25:2013–2021, 1986
52. Philipson KD, Ward R: Effects of fatty acids on Na^+-Ca^{2+} exchange and Ca^{2+} permeability of cardiac sarcolemmal vesicles. *J Biol Chem* 260:9666–9671, 1985
53. Koynova RD, Tenchov BG, Quinn PJ, Laggner P: Structure and phase behavior of hydrated mixtures of L-dipalmitoylphosphatidylcholine and palmitic acid: correlations between structural rearrangements, specific volume changes, and endothermic events. *Chem Phys Lipids* 48:205–214, 1988
54. Massey JB, Bick DH, Pownall HJ: Spontaneous transfer of monoacyl amphiphiles between lipid and protein surfaces. *Biophys J* 72:1732–1743, 1997
55. Weisiger RA, Ma WL: Uptake of oleate from albumin solutions by rat liver. *J Clin Invest* 79:1070–1077, 1987
56. Trigatti BL, Gerber GE: A direct role for serum albumin in the cellular uptake of long-chain fatty acids. *Biochem J* 308:155–159, 1995
57. Redelmeier TE, Hope MJ, Cullis PR: On the mechanism of transbilayer transport of phosphatidylglycerol in response to transmembrane pH gradients. *Biochemistry* 29:3046–3053, 1990
58. Hamilton JA, Bhamidipati SP, Kodali DR, Small DM: The interfacial conformation and transbilayer movement of diacylglycerols in phospholipid bilayers. *J Biol Chem* 266:1177–1186, 1991
59. Hamilton JA: ^{13}C-NMR studies of the interactions of fatty acids with phospholipid bilayers, plasma lipoproteins, and proteins. In *Carbon-13 NMR Spectroscopy of Biological Systems*. Beckman N, Ed. New York, Academic, 1994, p. 117–157
60. Ptak M, Egret Charlier M, Sanson A, Bouloussa O: An NMR study of the ionization of fatty acids, fatty amines, and *N*-acylamino acids incorporated in phosphatidylcholine vesicles. *Biochim Biophys Acta* 600:387–397, 1980

61. Miyazaki J, Hideg K, Marsh D: Interfacial ionization and partitioning of membrane-bound local anesthetics. *Biochim Biophys Acta* 1103:62–68, 1992
62. Traüble H: The movement of molecules across lipid membranes: a molecular theory. *J Membr Biol* 4:193–208, 1971
63. Homan R, Pownall HJ: Transbilayer diffusion of phospholipids: dependence on headgroup structure and acyl chain length. *Biochim Biophys Acta* 938:155–166, 1988
64. Kleinfeld A: Fatty acid transport across membranes. In *Permeability and Stability of Lipid Bilayers.* Disalvo EA, Simon SA, Eds. CRC, 1995, p. 241–258
65. Kleinfeld AM, Chu P, Romero C: Transport of long-chain native fatty acids across lipid bilayer membranes indicates that transbilayer flip-flop is rate limiting. *Biochemistry* 36:14146–14158, 1997
66. Stremmel W: Translocation of fatty acids across the basolateral rat liver plasma membrane is driven by an active potential-sensitive sodium-dependent transport system. *J Biol Chem* 262:6284–6289, 1987
67. Gutknecht J: Proton conductance caused by long-chain fatty acids in phospholipid bilayer membranes. *J Membr Biol* 106:83–93, 1988
68. Skulachev VP: Fatty acid circuit as a physiological mechanism of uncoupling of oxidative phosphorylation. *FEBS Lett* 294:158–162, 1991
69. Gutknecht J: Proton/hydroxide conductance and permeability through phospholipid bilayer membranes. *Proc Natl Acad Sci U S A* 84:6443–6446, 1987
70. Storch J, Kleinfeld AM: Transfer of long-chain fluorescent free fatty acids between unilamellar vesicles. *Biochemistry* 25:1717–1726, 1986
71. Doody MC, Pownall HJ, Kao YJ, Smith LC: Mechanism and kinetics of transfer of a fluorescent fatty acid between single-walled phosphatidylcholine vesicles. *Biochemistry* 19:108–116, 1980
72. Zhang F, Kamp F, Hamilton JA: Dissociation of long- and very-long- chain fatty acids from phospholipid bilayers. *Biochemistry* 35:16055–16060, 1996
73. Kamp F, Zakim D, Zhang F, Noy N, Hamilton JA: Fatty acid flip-flop in phospholipid bilayers is extremely fast. *Biochemistry* 34:11928–11937, 1995
74. Jezek P, Modriansk M, Garlid KD: Inactive fatty acids are unable to flip-flop across the lipid bilayer. *FEBS Lett* 408:161–165, 1997
76. Lisanti MP, Scherer PE, Vidugiriene J, Tang Z, Hermanowski Vosatka A, Tu YH, Cook RF, Sargiacomo M: Characterization of caveolin-rich membrane domains isolated from an endothelial-rich source: implications for human disease. *J Cell Biol* 126:111–126, 1994
77. Vork MM, Glatz JFC, van der Vusse GJ: On the mechanism of long-chain fatty acid transport in cardiomyocytes as facilitated by cytoplasmic fatty acid binding protein. *J Theor Biol* 160:207–222, 1993
78. Cabral DJ, Small DM, Lilly HS, Hamilton JA: Transbilayer movement of bile acids in model membranes. *Biochemistry* 26:1801–1804, 1987
79. Veith CM, Thalhammer T, Felberbauer FX, Graf J: Relationship of hepatic cholate transport to regulation of intracellular pH and potassium. *Biochim Biophys Acta* 1103:51–61, 1992
80. Bolhuis H, van Veen HW, Poolman B, Driessen AJ, Konings WN: Mechanisms of multidrug transporters. *FEMS Microbiol Rev* 21:55–84, 1997
81. Kleinfeld AM, Storch J: Transfer of long-chain fluorescent fatty acids between small and large unilamellar vesicles. *Biochemistry* 32:2053–2061, 1993
82. Smith R, Tanford C: Hydrophobicity of long-chain n-alkyl carboxylic acids, as measured by their distribution between heptane and aqueous solutions. *Proc Natl Acad Sci U S A* 70:289–293, 1973
83. Glatz JFC, Börchers T, Spencer F, van der Vusse GJ: Fatty acids in cell signaling: modulation by lipid binding proteins. *Prostaglandins Leukot Essent Fatty Acids* 52:121–127, 1995
84. Schaap FG, van der Vusse GJ, Glatz JFC: Fatty acid binding proteins in the heart. *Mol Cell Biochem* 180:43–51, 1998
85. Glatz JFC, Luiken JJFP, van Nieuwenhoven FA, van der Vusse GJ: Molecular mechanism of cellular uptake and intracellular translocation of fatty acids. *Prostaglandins Leukot Essent Fatty Acids* 57:3–9, 1997
86. Ho JK, Moser H, Kishimoto Y, Hamilton JA: Interactions of a very- long-chain fatty acid with model membranes and serum albumin: implications for the pathogenesis of adrenoleukodystrophy. *J Clin Invest* 96:1455–1463, 1995
87. Vork MM, Glatz JFC, van der Vusse GJ: Modelling intracellular fatty acid transport: possible mechanistic role of cytoplasmic fatty acid binding protein. *Prostaglandins Leukot Essent Fatty Acids* 57:11–16, 1997
88. van Nieuwenhoven FA, Verstijnen CPH, van Eys GJJM, van Breda E, de Jong YF, van der Vusse GJ, Glatz JFC: Fatty acid transfer across the myocardial capillary wall: no evidence of a substantial role for cytoplasmic fatty acid binding protein. *J Mol Cell Cardiol* 26:1635–1647, 1994
89. Vork MM, Glatz JF, van der Vusse GJ: Release of fatty acid binding protein and long-chain fatty acids from isolated rat heart after ischemia and subsequent calcium paradox. *Mol Cell Biochem* 123:175–184, 1993
90. Gericke A, Smith ER, Moore DJ, Mendelsohn R, Storch J: Adipocyte fatty acid binding protein: interaction with phospholipid membranes and thermal stability studied by FTIR spectroscopy. *Biochemistry* 36:8311–8317, 1997
91. Cooper R, Noy N, Zakim D: A physical-chemical model for cellular uptake of fatty acids: prediction of intracellular pool sizes. *Biochemistry* 26: 5890–5896, 1987
92. Noy N, Zakim D: Fatty acids bound to unilamellar lipid vesicles as substrates for microsomal acyl-CoA ligase. *Biochemistry* 24:3521–3525, 1985
93. Zakim D, Cooper RB, Noy N: The uptake of fatty acids by the liver. *Trans Am Clin Climatol Assoc* 99:41–46, 1987
94. Wolkowicz PE, Pownall HJ, McMillin Wood JB: (l-pyrenebutyryl)carnitine and 1-pyrenebutyryl coenzyme A: fluorescent probes for lipid metabolite studies in artificial and natural membranes. *Biochemistry* 21:2990–2996, 1982
95. Boylan JG, Hamilton JA: Interactions of acyl-coenzyme A with phosphatidylcholine bilayers and serum albumin. *Biochemistry* 31:557–567, 1992
96. DeGrella RF, Light RJ: Uptake and metabolism of fatty acids by dispersed adult rat heart myocytes. II. Inhibition by albumin and fatty acid homologues, and the effect of temperature and metabolic reagents. *J Biol Chem* 255:9739–9745, 1980
97. DeGrella RF, Light RJ: Uptake and metabolism of fatty acids by dispersed adult rat heart myocytes. I. Kinetics of homologous fatty acids. *J Biol Chem* 255:9731–9738, 1980
98. Luiken JJ, Turcotte LP, Bonen A: Protein-mediated palmitate uptake and expression of fatty acid transport proteins in heart giant vesicles. *J Lipid Res* 40:1007–1016, 1999
99. Van Nieuwenhoven FA, Luiken JJ, De Jong YF, Grimaldi PA, Van der Vusse GJ, Glatz JF: Stable transfection of fatty acid translocase (CD36) in a rat heart muscle cell line (H9c2). *J Lipid Res* 39:2039–2047, 1998
100. Harmon CM, Luce P, Beth AH, Abumrad NA: Labeling of adipocyte membranes by sulfo-*N*-succinimidyl derivatives of long-chain fatty acids: inhibition of fatty acid transport. *J Membr Biol* 121:261–268, 1991
101. Jochen A, Hays J: Purification of the major substrate for palmitoylation in rat adipocytes: N-terminal homology with CD36 and evidence for cell surface acylation. *J Lipid Res* 34:1783–1792, 1993
102. Febbraio M, Abumrad NA, Hajjar DP, Sharma K, Cheng W, Pearce SF, Silverstein RL: A null mutation in murine CD36 reveals an important role in fatty acid and lipoprotein metabolism. *J Biol Chem* 274:19055–19062, 1999
103. Ibrahimi A, Sfeir Z, Magharaie H, Amri EZ, Grimaldi P, Abumrad NA: Expression of the CD36 homolog (FAT) in fibroblast cells: effects on fatty acid transport. *Proc Natl Acad Sci U S A* 93:2646–2651, 1996
104. Stremmel W, Strohmeyer G, Borchard F, Kochwa S, Berk PD: Isolation and partial characterization of a fatty acid binding protein in rat liver plasma membranes. *Proc Natl Acad Sci U S A* 82:4–8, 1985
105. Berk PD: Point/counterpoint: how do long-chain free fatty acids cross cell membranes? *Proc Soc Exp Biol Med* 212:1–4, 1996
106. Berk PD, Wada H, Horio Y, Potter BJ, Sorrentino D, Zhou SL, Isola LM, Stump D, Kiang CL, Thung S: Plasma membrane fatty acid binding protein and mitochondrial glutamic-oxaloacetic transaminase of rat liver are related. *Proc Natl Acad Sci U S A* 87:3484–3488, 1990
107. Isola LM, Zhou SL, Kiang CL, Stump DD, Bradbury MW, Berk PD: 3T3 fibroblasts transfected with cDNA for mitochondrial aspartate aminotransaminase express plasma membrane fatty acid binding protein and saturable fatty acid uptake. *Proc Natl Acad Sci U S A* 92:9866–9870, 1995
108. Schaffer JE, Lodish HF: Expression cloning and characterization of a novel adipocyte long-chain fatty acid transport protein. *Cell* 79:427–436, 1994
109. Faergeman NJ, DiRusso CC, Elberger A, Knudsen J, Black PN: Disruption of the *Saccharomyces cerevisiae* homologue to the murine fatty acid transport protein impairs uptake and growth on long-chain fatty acids. *J Biol Chem* 272:8531–8538, 1997
110. Watkins PA, Lu JF, Steinberg SJ, Gould SJ, Smith KD, Braiterman LT: Disruption of the *Saccharomyces cerevisiae* FAT1 gene decreases very-long-chain fatty acyl-CoA synthetase activity and elevates intracellular very-long-chain fatty acid concentrations. *J Biol Chem* 273:18210–18219, 1998
111. Winkler E, Klingenberg M: Effect of fatty acids on H^+ transport activity of the reconstituted uncoupling protein. *J Biol Chem* 269:2508–2515, 1994
112. Garlid KD, Orosz DE, Modriansky M, Vassanelli S, Jezek P: On the mechanism of fatty acid–induced proton transport by mitochondrial uncoupling protein. *J Biol Chem* 271:2615–2620, 1996
113. Garlid KD, Jaburek M, Verecha M, Gimeno RE, Tartaglia LA: Transport function and regulation of uncoupling proteins 2 and 3 (Abstract). *Biophys J* 76:A1, 1999
114. Skulachev VP: Uncoupling: new approaches to an old problem of bioenergetics. *Biochim Biophys Acta* 1363:100–124, 1998
115. Klingenberg M, Huang S: Structure and function of the uncoupling protein from brown adipose tissue. *Biochim Biophys Acta* 1415:271–296, 1999
116. Klingenberg M: Mechanism and evolution of the uncoupling protein of brown adipose tissue. *Trends Biochem Sci* 15:108–112, 1990

117. Jezek P, Modriansk M, Garlid KD: A structure-activity study of fatty acid interaction with mitochondrial uncoupling protein. *FEBS Lett* 408:166–170, 1997
118. Schrauwen P, Walder K, Ravussin E: Human uncoupling proteins and obesity. *Obes Res* 7:97–105, 1999
119. Mao W, Yu XX, Zhong A, Li W, Brush J, Sherwood SW, Adams SH, Pan G: UCP4, a novel brain-specific mitochondrial protein that reduces membrane potential in mammalian cells. *FEBS Lett* 443:326–330, 1999
120. Hamilton JA, Civelek VN, Kamp F, Tornheim K, Corkey BE: Changes in internal pH caused by movement of fatty acids into and out of clonal pancreatic β-cells (HIT). *J Biol Chem* 269:20852–20856, 1994
121. Trigatti BL, Gerber GE: The effect of intracellular pH on long-chain fatty acid uptake in 3T3-L1 adipocytes: evidence that uptake involves the passive diffusion of protonated long-chain fatty acids across the plasma membrane. *Biochem J* 313:487–494, 1996
122. Mastrangelo AM, Jeitner TM, Eaton JW: Oleic acid increases cell surface expression and activity of CD11b on human neutrophils. *J Immunol* 161: 4268–4275, 1998
123. Esling C, Kassner A, Stremmel W: Effect of surface and intracellular pH on hepatocellular fatty acid uptake. *Am J Physiol* 271:G1067–G1073, 1996
124. Kleinfeld AM, Storms S, Wattes M: Transport of long-chain fatty acids across human erythrocyte ghost membranes. *Biochemistry* 37:8011–8019, 1998
125. Bröring K, Haest CW, Deuticke B: Translocation of oleic acid across the erythrocyte membrane: evidence for a fast process. *Biochim Biophys Acta* 986:321–331, 1989
126. Bojesen IN, Bojesen E: Specificities of red cell membrane sites transporting three long-chain fatty acids. *J Membr Biol* 149:257–267, 1996 (Erratum published in *J Membr Biol* 151:201, 1996)
127. Hamilton JA: Fatty acid transport: difficult or easy? *J Lipid Res* 39:467–481, 1998
128. Richieri GV, Ogata RT, Kleinfeld AM: The measurement of free fatty acid concentration with the fluorescent probe ADIFAB: a practical guide for the use of the ADIFAB probe. *Mol Cell Biochem*. In press
129. Darnell J, Lodish H, Baltimore D: *Molecular Cell Biology*. 2nd ed. New York, Freeman, 1990