review

Fatty acid transport: difficult or easy?

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Abstract Transport of unesterified fatty acids (FA) into cells has been viewed either as a simple diffusion process regulated mainly by lipid physical chemistry or as a more complex process involving protein catalysis. In this review FA transport in cell membranes is broken down into three essential steps: adsorption, transmembrane movement, and desorption. The physical properties of FA in aqueous, membrane, and protein environments relevant to transport mechanisms are discussed, with emphasis on recent information derived from NMR and fluorescence studies. Because of their low solubility in water and high hydrophobicity, FA bind rapidly and avidly to model membranes (phospholipid bilayers); if albumin is a donor, FA desorb rapidly to reach their equilibrium distribution between the membrane and albumin. The ionization properties of FA in a phospholipid bilayer result in a high population of the un-ionized form (~50%) at pH 7.4, which diffuses across the lipid bilayer (flip-flops) rapidly ($t_{1/2} < 1$ sec). Desorption of FA from a phospholipid surface is slower than transmembrane movement and dependent on the FA chain length and unsaturation, but is rapid for typical dietary FA. These physical properties of FA in model systems predict that proteins are not essential for transport of FA through membranes. The only putative FA transport protein to be purified and reconstituted into phospholipid bilayers, the mitochondrial uncoupling protein (UCP1), was shown to transport the FA anion in response to FA flip-flop. New experiments with cells have found that FA movement into cells acidifies the cytosol, as predicted by the flip-flop model.—**Hamilton, J. A.** Fatty acid transport: difficult or easy? *J. Lipid Res.* 1998. **39:** 467–481.

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INTRODUCTION

Unesterified fatty acids (FA) act in well-known capacities as intermediates in lipid metabolism and as fuel for cells. They are in constant flux and need to enter and leave cells rapidly. For example, well-oxygenated heart muscle cells demand more FA for fuel than can be supplied from the small triglyceride stores in these cells, and receive FA via the plasma compartment from adipocytes in distal sites (1). FA must leave the adipocytes, cross the capillary endothelium, travel through the blood stream, and eventually enter the cytosol of the heart cell. All these transport steps must be rapid on the time scale of cellular metabolism (minutes). FA also have important, more recently recognized, biological properties. For example, they can activate K^+ (2) and Ca^{2+} (3) channels in certain cells, influence the binding of low density lipoprotein to its receptor (4), reverse arrhythmias in cardiac myocytes in culture (5), and regulate expression of enzymes involved in their metabolism (6).

Transport of FA into cells, the subject of this review, is often vaguely defined, but minimally involves three distinct steps, beginning at the stage where FA is present in some unbound form, generally assumed to be the monomeric form, outside the cell. First, the FA must adsorb to the outer leaflet of the plasma membrane (adsorption). When the FA is present in the plasma or interstitial compartment, where it is bound to albumin, it must also desorb from albumin to enter the plasma membrane. Second, it must cross the membrane with accompanying re-orientation of the carboxyl head group to the cytosolic face (transmembrane movement). Third, it must leave the cytosolic leaflet if it is to be utilized in intracellular sites (desorption).

Each of these steps might be catalyzed by proteins in biological membranes, particularly if its rate is intrinsically slow. Hypothetical schemes, as reviewed (7, 8), have ranged from a single protein for any one of the steps to a multi-protein complex catalyzing all three steps. Often a protein transporter is postulated but the exact nature of the putative transporter is not de-

Abbreviations: BSA, bovine serum albumin; FA, fatty acid(s); FABP, fatty acid binding protein; HSA, human serum albumin; LUV, large unilamellar vesicles; SUV, small unilamellar vesicles; UCP, uncoupling protein.

scribed. A quite complex scheme (7) required enzymatic transformations of lipids to achieve transmembrane movement of FA. Is transport of FA inherently difficult and complicated? This question will be addressed by dissecting transport into each of its steps, understanding the physical chemical principles derived from simple model systems, and assessing the relevancy of such information to cell membranes.

ADSORPTION

The first step in the transport of FA across a membrane, adsorption of FA to the side of the membrane exposed to the FA, distinguishes the transport of these lipophilic molecules from that of other small molecules such as glucose. Glucose is highly soluble in water and partitions very weakly into lipid membranes, e.g., model membranes comprised of phospholipid bilayers suspended in an aqueous medium. In contrast, FA partition very favorably into phospholipids, creating a membrane highly enriched in FA compared to the surrounding aqueous environment. The very different physical properties of FA and glucose might lead one to predict that their transport mechanisms are fundamentally different, yet FA transport is usually seen in the same framework as transport of water-soluble molecules. For example, in interpreting FA uptake in cells, the concentration of unbound FA is generally used in data analysis and the concentration in the membrane is disregarded. Furthermore, until recently the values of unbound FA have been estimated from equilibrium constants for aqueous albumin (in the absence of membranes). New measurements of the concentration of unbound FA in the presence of albumin, which are probably more accurate, have shown much lower values (9). As a basis for predicting what transport mechanisms are required for FA in cells, we begin with biophysical studies of simple model systems and examine what the properties of FA imply about their transport.

The primary model system for FA transport that will be discussed is a unilamellar protein-free phospholipid vesicle, a single lipid bilayer encapsulating water (or buffer). Vesicles can be prepared with a diameter of ~250 Å by sonication (small unilamellar vesicles, SUV) or with larger diameters (large unilamellar vesicles, LUV) by other methods such as extrusion through pores or dialysis. Typically, vesicles are prepared with phosphatidylcholine (PC), the predominant phospholipid in most cell membranes, but can be prepared with mixed phospholipids and other lipids as minor components to model specific membranes. SUV have a highly curved surface with a small internal volume and can be prepared to a high degree of homogeneity. LUV have a lower surface curvature and larger internal volume; however, they can entrap smaller vesicles and have a higher variability in diameter.

The rate constant for adsorption of FA monomers to phospholipid bilayer vesicles is extremely fast. Knowledge of the affinity constant of a FA for a phospholipid bilayer, together with the rate constant for desorption from the membrane, allows calculation of the on rate constant ($K_{eq} = k_{off}/k_{on}$). As an example, consider binding of palmitic acid to dimyristoyl PC SUV. From the measured K_{eq} (4×10^6) and k_{off} ($6\ sec^{-1}$) (10, 11), the calculated K_{on} for adsorption of the monomer is $2.4 \times 10^7/s$. The actual rate of binding is likely to be diffusion limited, dependent on the concentration of FA monomers, and largely independent of the specific FA chain. As indicated by the affinity constant, FA not only adsorb rapidly to a phospholipid bilayer, they adsorb quantitatively, i.e., the partition coefficient lies heavily in favor of the lipid. This factor will favor rapid transport across the membrane, provided that the FA molecule can pass through interior of the bilayer, as discussed below.

Many experiments in which FA are added to model membranes or cells are carried out above the monomeric solubility limit of the FA. At pH values found in most physiological environments (pH = 7.0–7.4), long chain FA do not, as often assumed, form soluble micelles. Instead, their equilibrium state is an acid–soap bilayer, an insoluble aggregate much like the phospholipid bilayer (12, 13). The solubility limit of the monomeric FA is very difficult to measure; for palmitic acid it is generally considered to be in the μm range but has been estimated to be as low as 0.1 nm at pH 7.4 (14). To achieve higher concentrations for presentation to membranes, FA can be prepared in a soluble micellar form as Na^+ or K^+ salts at high pH or as an ethanolic solution of the acid. Alternatively, they can be presented in a bound form, which is in equilibrium with a low concentration of unbound FA. In both cases the observed rate of uptake may be influenced by the kinetics of adsorption. We have found in stopped flow fluorescence experiments that binding of typical dietary FA presented in the micellar form at concentrations of ~10 μm to SUV at pH 7.4 is faster than the mixing time of the instrument (5–10 ms), showing that the FA bind to the vesicles before they precipitate as the acid–soap. However, with saturated FA of >18 carbons, it is difficult even to prepare soluble micelles at desired concentrations, and the kinetics of binding to PC vesicles are clearly limited by the dissolution of aggregates (15). Thus, studies of FA transport in cells or in more complex physiological systems carried out without albumin may be subject to artifacts because FA form aggregates

under typical experimental conditions. Some fraction of the FA may bind rapidly while the remaining fraction binds slowly, giving rise to biphasic or complex kinetics. In this regard, precautions must be made in designing and interpreting experiments comparing long chain FA with the more water-soluble medium chain FA.

The very low aqueous solubility of long chain FA can be overcome by binding to serum albumin, which allows high (up to mm) concentrations of FA to circulate in the plasma in a non-aggregated form that is readily available to cells (7). By using FA/albumin complexes as a source of FA in transport studies, the FA is maintained in a non-aggregated form, but other complications are introduced. In protocols where FA are added alone (as micelles) or in a solvent such as ethanol, they will either bind to the membrane or precipitate. When albumin is present, the relative affinities of albumin and the membrane for the specific FA, which determines the partitioning, must be considered. In experiments with cells, it is difficult to determine the fraction of FA that desorbs from albumin to bind to the outer leaflet of the plasma membrane to achieve this partitioning before any other transport or metabolic process occurs.

Because of the high affinity of albumin for FA, it might be assumed that FA cannot leave binding sites on albumin without catalysis by another protein. In fact, studies of FA uptake in perfused liver led to the notion of an albumin receptor on the liver plasma membrane (16). The receptor hypothesis arose primarily from the appearance of the uptake curve for oleic acid, which showed an apparent saturation at higher concentrations of FA. The key physical chemistry questions are whether *i*) the affinity of albumin for FA is too high compared to a simple phospholipid bilayer and/or *ii*) the rate of desorption from albumin is too slow to allow appreciable transfer of FA. While net transfer of FA to phospholipid bilayers (17) or to cells (18, 19) can be measured by methods that involve separation of donor and acceptor (possibly affecting the binding equilibrium), it is not trivial to make such a demonstration in a complex mixture without separation procedures.

With ^{13}C NMR spectroscopy, it is possible to observe binding of FA to albumin or to phospholipid bilayers by characteristic chemical shifts of the carboxyl carbon. Long chain FA show several resonances in the presence of albumin, reflecting heterogeneous binding sites (20, 21), and a single resonance in the presence of phospholipid bilayers (22, 23). The chemical shift of FA in the bilayer is strongly dependent on pH in the range of physiological pH values. As illustrated in **Fig. 1**, the binding of FA to both albumin and PC bilayers can be observed in a mixture of albumin and vesicles; the peak positions identify where the FA is bound, and the peak

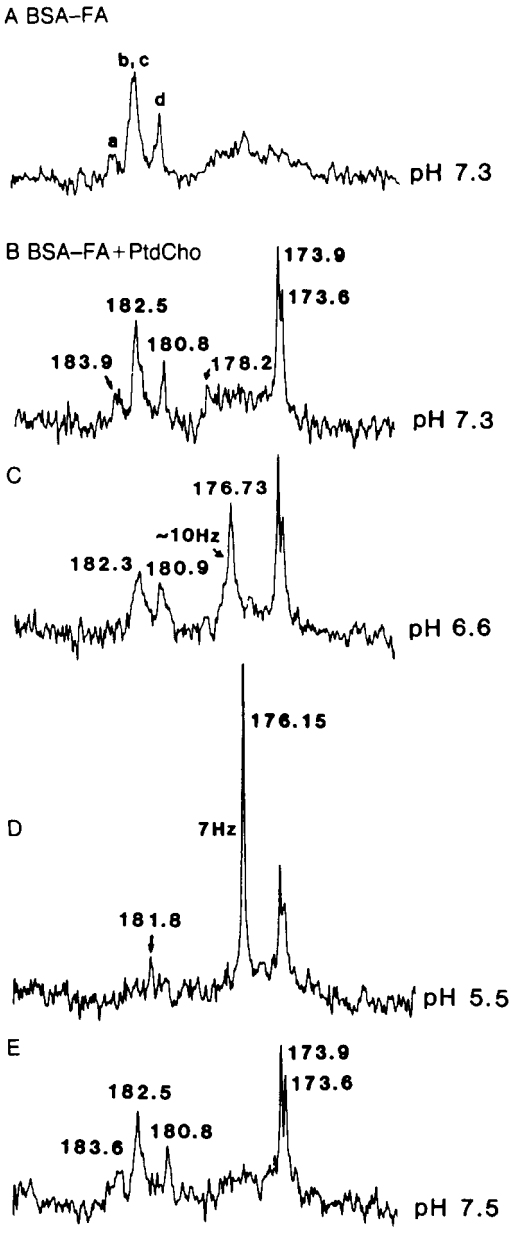

Fig. 1. pH-dependent partitioning of FA between model membranes (phospholipid vesicles) and albumin (BSA). The carboxyl and carbonyl region of the ^{13}C NMR spectrum at 35°C of (A) 4 mol of 90% [1-^{13}C]oleic acid per mol of BSA at pH 7.3; (B) a 50:50 (vol/vol) mixture of the BSA-FA complex from (A) and egg phosphatidylcholine vesicles containing no oleic acid at pH 7.3, and the same mixture at pH 6.6 (C), pH 5.5 (D), and pH 7.5 (E). All spectra were recorded after 2000 accumulations at a pulse interval of 1.8 sec. The letter designations in A describe the major peaks of the oleic acid/albumin complex. The number given above each peak is the chemical shift in ppm; line width values of selected peaks in C and D are indicated in Hz. (Reproduced with permission from Hamilton, J. A., and D. P. Cistola. 1986. Transfer of oleic acid between albumin and phospholipid. *Proc. Natl. Acad. Sci. USA.* **83:** 82–86. Copyright 1986 National Academy of Sciences, USA.)

intensities report how much is bound in each environment, i.e., the partitioning. These spectra show a reversible transfer of oleic acid, the most "tightly" bound FA to albumin (24), between albumin and phospholipids, and demonstrate clearly that there is no a priori need for an additional protein to partition FA away from albumin. Moreover, the experiments of Fig. 1 demonstrate that bulk pH is a simple but potent "mechanism" for altering the amount of FA partitioning into the membrane. Thus, basic physical chemistry may explain the enhanced "uptake" of FA into cells when the pH of the medium is decreased (25).

From NMR data as in Fig. 1, partition coefficients can be derived to allow prediction of the partitioning with different amounts of albumin and model membranes. Partition coefficients have also been derived from fluorescence experiments on model systems (10, 11, 26). Additional pH-dependent partitioning data derived from ^{13}C NMR experiments with lipoproteins, albumin, and phospholipid vesicles has permitted quantitative predictions of how FA will distribute among these pools under different physiological conditions (27). The pH-dependent partitioning data, for example, allow predictions of how FA distribution will be altered in acidosis. This kind of quantitative data for biological membranes is rarely available but is valuable for predicting how FA distributes between albumin and cells and for estimating the steady state concentration of FA in the cell membranes (28).

^{13}C NMR data for oleic acid transfer between albumin and vesicles revealed a time range for exchange of 0.1 sec $< t_{1/2} <$ min (22). The actual exchange rate could not be measured. However, for medium chain FA it was possible to derive kinetic data from NMR experiments because the faster exchange fell into a time range suitable for measurement (29). The $t_{1/2}$ values representing the rate-limiting step (most likely desorption from albumin) for exchange of octanoic acid and decanoic acid were very fast (2 msec and 35 msec, respectively, at 32–33°C).

Fluorescence studies have also added greatly to our understanding of the mechanisms, thermodynamics, and kinetics of FA transport. A general strategy of fluorescence experiments is to complex the FA with a donor molecule or aggregate and monitor its transfer to an acceptor by a fluorescent group in the donor or acceptor. This strategy allows study of natural FA rather than fluorescent-labeled FA, and the measurements are made without separation of donor and acceptor. For the specific issue of how FA that is bound with very high affinity to albumin can be transferred to membranes, the rates of desorption of long chain FA from albumin have been measured by monitoring the intrinsic fluorescence of albumin, which is affected by binding of FA. The desorption rate was dependent on the chain length and saturation of the FA; the half time for the first-order process, which essentially consists of hydration of the FA, varied from 6 msec for myristic acid to 70 sec for stearic acid at 37°C (11). Rate constants obtained by ^{13}C NMR for transfer of octanoic and decanoic acids between vesicles and BSA (29) were on a time scale predicted by linear extrapolation of values of desorption of long chain FA from albumin. Thus, despite their high affinity binding to albumin, FA can leave the binding sites rapidly.

More recent measurements in our laboratory using pyranin fluorescence (see below) have suggested that desorption rates of long chain FA from BSA are somewhat faster than those cited above (26). However, our donor FA/albumin complexes had higher FA/protein ratios, and we have not made a systematic study with lower ratios. Transfer of long chain FA from a fluorescent-labeled human serum albumin (HSA) to native HSA was also faster; for example the $t_{1/2}$ for stearic acid was 1 sec (30). Nevertheless, even the slower rates reported by other groups (11, 31) are sufficiently fast to allow the conclusion that spontaneous desorption of FA can account for tissue or organ uptake of FA and that catalysis of desorption by another protein is not needed (28). One of the original proponents of receptor-mediated uptake of FA in the liver re-investigated the issue and found no evidence that the liver catalyzes desorption of FA from albumin (32). Very significantly, the FA uptake curve (rate of uptake vs. concentration of FA), which has been used by many investigators to invoke protein-mediated uptake at some stage of FA incorporation into cells, can be explained by the partitioning of FA between albumin and membranes (33). It must be kept in mind that long chain FA have a very limited solubility compared to molecules like glucose, and the kinetics are complicated by the physical chemistry and binding properties of FA.

The adsorption step is now probably the least controversial aspect of FA transport, although there are still some proponents of complex mechanisms for FA transfer from albumin (34). Even if specific mechanisms are not required to release FA from albumin, FA adsorption to cell membranes must be more complex than adsorption to simple phospholipid bilayers. Extracellular components such as the collagen matrix surrounding adipocytes could decrease the rate at which an FA molecule reaches the plasma membrane. Non-lipid components of the membrane and the unstirred water layer (35) may also decrease the rate at which an FA molecule reaches the lipid components. In addition to the kinetic aspects of adsorption, the thermodynamics of FA interactions as reflected in partitioning are important in FA transport. The partitioning of FA between al-

bumin and the plasma membrane will be affected by factors such as the FA to albumin ratio, the type of FA, the relative amounts of membrane and albumin, the membrane composition, the pH, and temperature. These physical chemical factors will thus affect the transport of FA into the cell by modulating the concentration of FA in the plasma membrane.

TRANSMEMBRANE MOVEMENT

The most controversial aspect of FA transport has been the transmembrane step, and the controversy has extended even to simple model systems. Can FA diffuse passively (flip-flop) across a phospholipid bilayer at rates sufficient to supply cells with FA, or are other mechanisms required? The essential problem is to place an FA molecule on one side of a bilayer membrane and determine how long it takes to reach the other side, and this is not a trivial task, especially with a simple molecule lacking an intrinsic probe.

The issue of flip-flop has been approached by measuring rates of transfer of FA from phospholipid vesicles by fluorescence methods, often with fluorescent-labeled FA. Transfer of pyrene-labeled FA was shown to exhibit a single exponential curve, and this rate-limiting step was attributed to desorption (36). The kinetics of anthroyloxy (AO) FA transfer were interpreted to indicate that flip-flop was slow compared to desorption (37, 38), the opposite conclusion of the first study. The flip-flop of ionized AO FA was inferred to be faster than that of the un-ionized form (37). A study of transfer of natural FA from vesicles to albumin concluded that flip-flop was fast compared to desorption and probably occurred on the msec time scale (11). It was assumed (11) that FA was present initially in both leaflets rather than only the outer leaflet, requiring flip-flop to allow extraction of all the FA in donor vesicles, but flip-flop was not measured directly. The AO FA studies (37, 38) provided evidence that AOFA was distributed in both bilayer leaflets but attributed the slower step of a bi-exponential transfer process to flip-flop.

In considering the mechanisms of passive and facilitated diffusion, it is important to understand the ionization properties of FA in membranes. It has often been assumed (without evidence) that FA are present in a membrane exclusively as anions; the logical extension of this premise is that diffusion of FA across membranes must be slow without an anion transporter (39). Indeed, a perceived difficulty with the diffusion hypothesis is that it does not explain how the FA anion crosses the hydrophobic barrier of the lipid bilayer (7). Is the FA fully ionized at physiological pH, or is there a population of un-ionized FA in the membrane? The ionization state of the FA in a membrane (not of the FA in solution) must be measured to answer this question. This can be done by very straightforward ^{13}C NMR experiments. FA with [^{13}C]carboxyl-enrichment are incorporated into phospholipid vesicles (SUV), and the carboxyl chemical shift is monitored as a function of pH to construct a titration curve of the FA in the membrane environment. Several investigators have shown that the apparent pK of the FA in SUV (~7.5) is close to physiological pH (22, 40, 41). The FA carboxyl group lies at the aqueous interface, and the large shift to higher pK is predicted from its proximity to the negative charges of the phospholipid (42). The pK is independent of the FA chain length (8–26 carbons) and is slightly affected by the phospholipid composition and cholesterol content of the model membrane (23). To address the general relevancy of the SUV model systems for biomembranes, we have also measured apparent pK values of ^{13}C-enriched oleic acid incorporated into phospholipid multilayers (23) and plasma membranes (W. Guo and J. A. Hamilton, unpublished results); in both cases the pK was ~7.5. These NMR results thus show that the charged surface of a membrane affects the ionization of a FA, increasing the apparent pK significantly. In the range of usual physiological pHs, about 50% of the FA are un-ionized.

Does the phospholipid bilayer in cell membranes present a barrier to transmembrane movement of FA? Clearly, FA bind rapidly and with high affinity to phospholipid bilayers, which could facilitate their movement into cells. From knowledge of their ionization behavior in a membrane, it can be hypothesized that FA could diffuse across the lipid bilayer via their un-ionized form. This hypothesis was tested in our laboratory with a new fluorescence approach which: *i*) allowed measurements of natural FA; *ii*) did not require interaction of the fluorophore with the FA or the lipid bilayer; and *iii*) measured the transmembrane step more directly than existing methods (most important, it did not require desorption of the FA molecule from the lipid bilayer). The experimental protocol is to add FA in a soluble form to SUV or LUV and monitor the pH inside the vesicle by an entrapped pH-sensitive fluorophore, pyranin.

If oleic acid (K^+ oleate) is added to egg PC SUV with entrapped pyranin, the fluorescence of pyranin decreases immediately (within the dead time of the fluorometer, ~1 sec), as shown in **Fig. 2**. The decrease in pH is explained by the movement of un-ionized FA to the inner leaflet of the vesicle, followed by ionization of some of the FA, and diffusion of the proton to pyranin in the aqueous phase (43). The pH then begins to return to equilibrium slowly from leakage of protons through the bilayer. When albumin is added to the

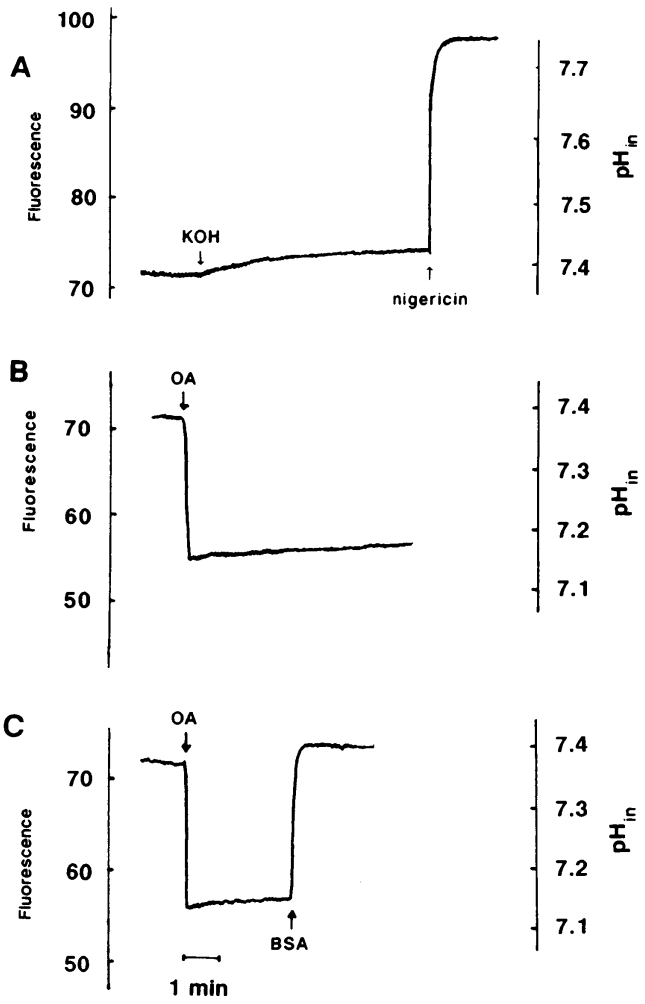

Fig. 2. Rapid flip-flop of un-ionized FA in vesicles. (A) Addition of KOH, followed by nigericin, to SUV with trapped pyranin. pH_{out} was measured with a pH electrode, and the pH_{in} by fluorescence of pyranin. At the beginning, $pH_{in} = pH_{out} = 7.4$. Upon KOH addition, a pH gradient of 0.35 unit was established, which relaxed very slowly until nigericin was added, when $pH_{in} = 7.73$. (B) Effect of adding 20 nmol of oleic acid (OA) (1.5 mol % with respect to phospholipid) as potassium oleate on the pyranin fluorescence of SUV. The pH_{in} decreased by 0.22 unit; pH_{out} remained at 7.38. (C) Addition of OA, followed by BSA, to vesicles with trapped pyranin. Addition of 20 nmol of OA caused pH_{in} to drop from 7.35 ± 0.03 to 7.13 ± 0.03. Addition of 10 nmol of BSA 3 min later instantly increased pH_{in} to 7.38 ± 0.03. (Reproduced with permission from Kamp, F., and J. A. Hamilton. 1992. pH gradients across phospholipid membranes caused by fast flip-flop of un-ionized fatty acids. *Proc. Natl. Acad. Sci. USA.* **89:** 11367–11370. Copyright 1992 National Academy of Sciences USA.)

buffer to partition the FA from the vesicle (the proportion to achieve complete removal of FA can be estimated from NMR experiments), the pH immediately returns to equilibrium. Because only the un-ionized FA can move rapidly through the bilayer, the protons originally donated must be reclaimed. The rapid rise in pH also implies that desorption of oleic acid is fast (see below). FA generated directly within the bilayer by phospholipase A_2 hydrolysis of phospholipid also exhibited rapid flip-flop (26).

Our model of passive diffusion by flip-flop (43) is shown in **Fig. 3**. Upon addition of FA to a suspension of vesicles, FA bind rapidly to the outer leaflet of the bilayer, where they quickly reach ionization equilibrium to establish a population of ~50% ionized, 50% un-ionized. This pK represents an equilibrium state and is attained irrespective of the form in which FA is added: as the acid in ethanol or another organic solvent, as the anion in a high pH solution, or as a FA/albumin complex. The un-ionized FA move across the bilayer rapidly compared to the ionized FA in response to concentration gradient. Upon reaching the inner bilayer, which is exposed to the pH 7.4 buffer, half of the newly arrived un-ionized FA molecules ionize and contribute protons to the buffer containing pyranin. As predicted by the theory: *i*) higher amounts of FA produce larger pH decreases; *ii*) acidification of the internal aqueous volume suppresses the net movement of FA to the inside; *iii*) pH changes in the internal volume match the calculated pHs for both SUV and LUV; and *iv*) a lower buffer capacity yields a larger pH decrease (15). As shown in **Fig. 3**, the anion flip-flop is very slow, and cyclical transport of protons does not occur in the phospholipid bilayer. The model is general and predicts that a charged amphiphilic molecule might be capable of fast flip-flop if there is an appreciable fraction of the uncharged form in the bilayer. Pyranin experiments have shown that FA from 8–26 carbons in length, certain bile acids, retinoic acid, and long chain amines exhibit rapid flip-flop in SUV (26). In the case of the amines, inward movement is accompanied by a pH increase, as predicted.

The first measurements of FA flip-flop by the pyranin fluorescence response were limited by the mixing time and response time of the fluorometer, ~1–2 sec. The FA-promoted pH decrease was complete within this time, giving an upper limit for the rate of flip-flop of $t_{1/2} = 1$ sec. To assess whether flip-flop is actually faster than this, we monitored FA movement in SUV and LUV by stopped flow experiments, with a fluorometer response time of 1 msec and a mixing time of 5–10 msec (15). For the FA studied (14:0, 16:0, 18:0, 18:1), the pH decrease in SUV was complete within the mixing time. In LUV the pH decrease followed a single exponential decay with $t_{1/2} \sim 35$ msec; there was no apparent dependence of the $t_{1/2}$ (±50%) on FA chain length in this series of FA. In the LUV experiments, we have not excluded the possibilities that the rate of adsorption of FA to LUV or the rate of proton diffusion to pyranin in the large inner volume contribute to the measured rate process. Nevertheless, it can be con-

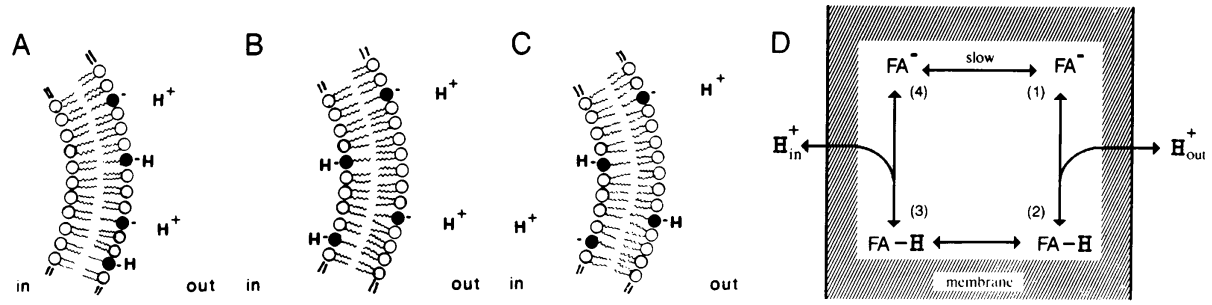

Fig. 3. Schematized mechanism of FA movement across bilayers. (A) Four FA molecules bound at the outer surface of a vesicle. Fifty percent of the FA molecules are ionized. (B) The un-ionized FA flipped to the inner leaflet. (C) Fifty percent of the FA molecules in both leaflets are ionized. The protons released inside the vesicle cause a measurable drop in pH. (D) Kinetic diagram. FA bound to the membrane of SUV can be in four states: the head group can be ionized or un-ionized (protonated), at either the inner or outer surface. The horizontal transitions represent the flip-flop reactions. The vertical transitions are the (de)protonation reactions. All transitions are reversible. (Reproduced with permission from Kamp, F., and J. A. Hamilton. 1992. pH gradients across phospholipid membranes caused by fast flip-flop of un-ionized fatty acids. *Proc. Natl. Acad. Sci. USA.* **89:** 11367–11370. Copyright 1992 National Academy of Sciences USA.)

cluded that the rate of transbilayer movement is extremely fast for both SUV ($t_{1/2} \leq 10$ msec) and LUV ($t_{1/2} \leq 35$ msec).

We also have assessed the flip-flop of a less abundant group of FA, the very long chain saturated FA (VLCFA). These FA with >18 carbons play key roles in inherited diseases of FA metabolism, such as adrenoleukodystrophy (44). Because of their very low solubility (aqueous or in ethanol), they could not be studied by the experimental protocol of adding the FA to vesicles in buffer. Another way to monitor flip-flop is to prepare vesicles with FA in the sonication mixture, a procedure that takes advantage of the solubility of FA in phospholipids, and that creates an initial state in which the FA is distributed in both leaflets of the bilayer. The distribution of FA in the two leaflets can then be perturbed by altering the pH of the external buffer and creating a pH gradient across the membrane. If the pH_{out} is increased, there will be a (small) net redistribution of FA to the outer leaflet, accompanied by a pH increase inside the vesicle. With this protocol we showed that flip-flop of saturated VLCFA with 20–26 carbons in SUV takes place within 1–2 sec, the time resolution of the fluorescence measurement.

Fast flip-flop of FA shown by the pyranin assay is consistent with rates reported for FA and other lipophilic molecules by other methods. Most of these methods perturbed the equilibrium distribution of the molecule in a vesicle and measured the re-equilibration or the relaxation to equilibrium. The highest time resolution measurements have been made using a rapid temperature jump to induce a pH gradient in vesicles containing pyranin and monitoring the response on a μsec time scale (45). Octanoic acid showed transbilayer movement in vesicles with an apparent rate constant of 15×10^3 sec^{-1} ($t_{1/2} = 50$ μsec) at 37°C in response to the pH gradient, and comparable rates were found for alkylamines. The rate constant showed less than an order of magnitude increase between the C-4 and C-12 amine (45). By using Ca^{2+} to alter the transbilayer distribution, other investigators showed that oleic acid moved across SUV bilayers with $t_{1/2} < 1$ sec (46). NMR measurements have shown that unconjugated bile acids flip-flop across PC SUV via the un-ionized form with $t_{1/2}$ as low as 5 msec. Cholic acid in SUV at pH 7.4 showed a slower flip-flop ($t_{1/2} = 12$ sec) by the pyranin assay than at pH 3.0 ($t_{1/2} = 140$ msec) by the NMR measurement (47). Flip-flop of a diacylglycerol in SUV was shown by NMR to occur very rapidly at 38°C ($t_{1/2} = 10$ msec). The rapid transbilayer movement of larger molecules with a greater number of polar groups implies that FA movement could be even more rapid, consistent with estimates of msec or faster. Data for one type of FA analogue, anthroyloxy FA, may be in conflict with the above generalizations. Based on fluorescence experiments monitoring transfer of AOFA from phospholipid vesicles measuring both flip-flop and desorption, it has been concluded that AOFA flip-flop is slow; the pseudo first order rate constant in SUV (38) is ~1000-fold lower than that of a natural FA (15). Recent measurements in our laboratory using somewhat different protocols, including the response of pyranin fluorescence to an imposed pH gradient in SUV pre-loaded with 12-AO stearic acid, suggested that AOFA flip-flop is fast (15). While there is disagreement about the rate of flip-flop of AOFA, if the rate is slow, the AOFA is not a good model for the kinetics of FA flip-flop or cellular uptake.

In marked contrast to unesterified FA, the activated metabolic derivatives of FA, acyl coenzyme A and acyl carnitine, do not move across a phospholipid bilayer

spontaneously. The distribution of these molecules in the two leaflets of a phospholipid bilayer (SUV) can be determined by ^{13}C NMR spectroscopy of the carbonyl-enriched lipids. When acyl coenzyme A (48) or acyl carnitines (49) are added to SUV, a signal representing the lipid in the outer leaflet is seen immediately. Even after 24 h, no movement of lipid to the inner leaflet is detected. Therefore, the difference in flip-flop rates compared to FA must be many orders of magnitude. Both acyl coenzyme A and acyl carnitines have negative charges at physiological pHs that are not neutralized in the phospholipid environment, and these molecules are not expected to permeate the bilayer. Thus, simple physical chemistry predicts that both molecules will require a specialized transport mechanism to carry them across a membrane. This prediction is consistent with the well-known complex mechanism for entry of acyl coenzyme A into the beta-oxidation pathway in mitochondria, which involves enzymatic transformations and protein transporters (50). Furthermore, the conversion of FA to acyl coenzyme A or acyl carnitine serves to trap the FA inside the cell.

DESORPTION

While it is evident from the high affinity of FA for phospholipid bilayers (large association constant) that the rate constant for desorption must be much lower than that for association, it has been debated whether desorption is slower than flip-flop. It has also been speculated that the abundant intracellular protein family, fatty acid binding protein (FABP), might be required to facilitate transfer of FA from the cytosolic surface of the plasma membrane to the aqueous cytosolic compartment (51). Because of technical limitations, there have been few measurements of desorption reported for natural FA. Furthermore, desorption of FA from model membranes has generally been measured indirectly by monitoring transfer of FA from a donor vesicle to other vesicles or to albumin. Thus, the measurement includes the flip-flop and desorption steps in the donor (and sometimes the adsorption step in the acceptor), and assumptions about the kinetics of each step must be made if the steps are not measured separately.

The pyranin method originally used to monitor flip-flop can be adapted to measure transfer of FA from donor vesicles to an acceptor. With knowledge of the flip-flop rates (or upper limits thereof) measured by the same method, the kinetics of desorption can be reliably quantified. One protocol for desorption measurements is to incorporate FA into vesicles that also contain trapped pyranin; transfer to FA-free vesicles or BSA will be accompanied by a pH increase in the inner volume of the donor vesicles. Another protocol is to prepare vesicles with FA and then add FA-free vesicles with trapped pyranin, in which case an acidification will be recorded as FA bind to the acceptor vesicles and flip-flop to the inner leaflet.

To derive rate constants for desorption of FA from phospholipid vesicles, we measured the transfer kinetics for FA with chain lengths of 14 to 26 carbons (52). Because of the wide time range, it was necessary to monitor the kinetics by stopped flow (C14:0 to C18:0, C18:1; C18:2) and on-line (C20:0 to C26:0) fluorescence and NMR spectroscopy (C26:0). The transfer kinetics for each FA followed a single exponential pro-

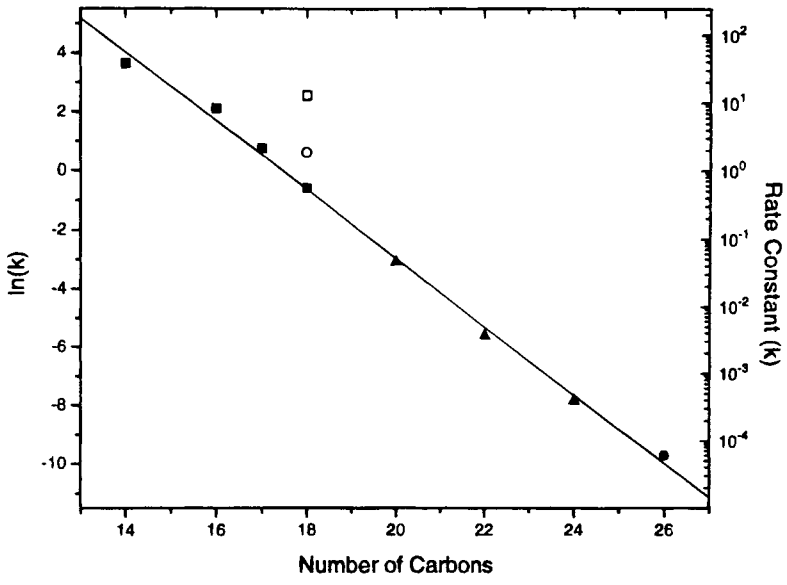

Fig. 4. Desorption of FA from SUV. Plot of 1n (k_{off}) at 24 ± 1°C versus FA chain length. Solid symbols are for saturated FA chain lengths of 14–26 carbons. The k_{off} values for C14:0, C16:0, C17:0, and C18:0 (solid squares), C18:1 (open circle), and C18:0 (open square) were obtained by transfer studies using stopped flow fluorescence; the k_{off} values for C20:0, C22:0, and C22:0 (solid triangles) were obtained by on-line fluorometry. The k_{off} for C26:0 (solid circle) was previously measured by ^{13}C NMR methods (54). (Reproduced with permission from *Biochemistry.* 1996. **35:** 16055–16060.)

cess. The calculated rate constant was always lower than the upper limit for flip-flop previously determined for the specific FA and was attributed to the desorption step. As shown in **Fig. 4**, the pseudo-unimolecular rate constants (k_{off}) for the rates of desorption of FA with <20 carbons are fast ($t_{1/2} < 1$ sec). Saturated FA with 14 to 26 carbons followed a very predictable trend with chain length. There is ~ a 10-fold increase in the rate constant for addition of two CH_2 groups to the aliphatic chain, and ~ a 5-fold decrease for each double bond in the 18-carbon chain. The activation energy per CH_2 group calculated from Fig. 4 (−740 cal) is close to the value derived for the partitioning of long chain FA and other related lipids between an organic phase and water (53). Thus, the kinetics of desorption are determined by the thermodynamics of partitioning, and the "fear of water" (hydrophobia) is the deterrent for FA desorption from vesicles. Although it has been suggested that the intracellular FABPs may facilitate desorption of normal dietary FA (51), the very long chain fatty acids would seemingly benefit more from such a mechanism. However, these FA accumulate in cell membranes where they cause damaging effects. VLCFA bind more weakly to albumin than long-chain FA (54); to our knowledge, binding of saturated VLCFA (e.g., C20:0–C26:0) to FABP has not been studied.

The fluorescence data for the saturated FA with 14–18 carbons are in excellent quantitative agreement with previous data for transfer of these FA from a different donor, dimyristoyl PC (11). In addition, new fluorescence studies have supported these results and conclusions (30). The transfer of saturated and unsaturated FA with 16–22 carbons was monitored by the fluorescence of a labeled albumin, anthraniloyl-HSA (an-HSA). **Figure 5A** shows a single exponential increase in the an-HSA fluorescence after phospholipid vesicles with palmitic acid were added. This process, which must be either flip-flop or desorption, was attributed to desorption from the bilayer and follows a time course similar to that found by us for transfer of palmitic acid between phospholipid vesicles (52). In an experiment where flip-flop is not a factor (Fig. 5B), the an-HSA fluorescence showed a single exponential decrease when FA desorbed from the an-HSA to binding sites on FA-free HSA. The data for desorption of various mono- and polyunsaturated FA showed strong correlations with chain length, as for the saturated FA. The free energy of activation (calculated from temperature-dependent data) plotted versus chain length and unsaturation of the FA (**Fig. 6**) shows quantitatively how each methylene unit and double bond contributes to the free energy of activation. The kinetics of desorption of very long chain polyunsaturated FA fall into a faster time range than their saturated counterparts, as observed previously in our study with a more limited selection of unsaturated FA (52). In addition, with the use of different donors and acceptors, it was clearly shown that the length and unsaturation of the FA are more important determinants of the kinetics than the type of donor vesicle or acceptor (30). This work provides additional evidence that desorption is a spontaneous, first order process controlled in a predictable way by thermodynamics.

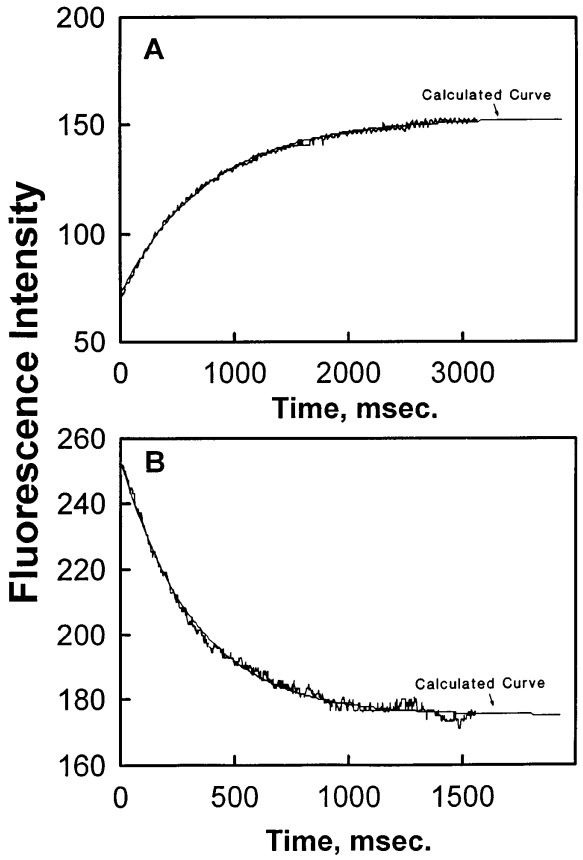

Fig. 5. Kinetics of the transfer of palmitic acid from SUV to an-HSA (A) and from an-HSA to HSA (B). (A) A representative kinetic trace following the mixing of 12 μm an-HAS with 40 μm palmitic acid (3 mol % in SUV) at 10°C. Analysis of the data according to first-order kinetics gave the solid line that is superimposed on the experimental data and rate constant, $k = 1.25 \pm 0.01\ s^{-1}$. (B) Kinetics of transfer of palmitic acid from an-HSA (15 μm) + palmitic acid (45 μm) to HSA (7.5 μm) at 30°C monitored from the decrease in an-HSA fluorescence. Analysis according to (A) gave a rate consistent, $k = 3.0 \pm 0.05\ s^{-1}$. (Reproduced with permission from *Biophys. J.* 1997. **72:** 1732–1743.)

RELEVANCE TO CELL PHYSIOLOGY

Theoretical considerations

Are FA "loose cannons" in the cell, passing freely through the plasma membrane and intracellular mem-

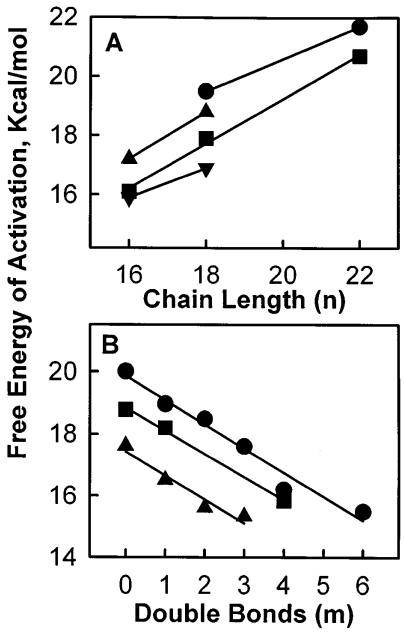

Fig. 6. Effects of amiphiphile structure on free energy of activation for transfer at 37°C. Effect of acyl chain length on the free energy of activation for the transfer of saturated amphiphiles from 1,2-dipalmitoyl-PC (DPPC) SUV to an-HSA (λ); 1-palmitoyl-2 oleoyl-PC (POPC) SUV to an-HSA (■); and an-HSA to HSA (●); and of *lyso*PCs from POPC SUV (▼); to an-HSA. (B) Effect of acyl chain unsaturation on the free energy of activation for the transfer of FA from POPC SUV to an-HSA, FA chains contained 22 (●); 20 (■); and 18 carbon atoms (▲); or from DPPC SUV to an-HSA (▼). (Reproduced with permission from *Biophys. J.* 1997. **72:** 1732–1743.)

branes without specific transporters, and diffusing to any intracellular site? Might FA reach the inside of the mitochondrion by simple diffusion and flip-flop? Or do the complexities of cells alter the rates of the various steps of transport drastically, requiring protein(s) at one or more steps to catalyze the rate?

The physical chemistry studies of natural FA and model membranes have provided detailed and quantitative data for the various steps of FA transport in simplified systems. Typical dietary FA exhibit rapid and spontaneous transport without proteins. FA bind very rapidly to a phospholipid bilayer to establish a high concentration relative to the aqueous phase and reach an ionization equilibrium characterized by pKa of ~7.5. They move spontaneously to the leaflet with the lower concentration of FA and desorb from the membrane at a rate that is highly dependent on the FA structure. If FA/albumin complexes are the source of FA in the external buffer, the equilibrium between membrane-bound and albumin-bound FA will be reached quickly.

The physical chemical model also provides a viable model of reversible transport. FA must not only enter a cell but also exit it rapidly. Adipocytes release FA from intracellular storage droplets for fuel in distal sites of the body. Under ischemic conditions, FA accumulate in cells, and transport out of the cell would alleviate cell damage. FA must therefore quickly and efficiently accomplish all of the transport steps in the reverse sense of FA influx into a cell. The physical processes of adsorption to the lipid membrane, flip-flop, and desorption are mechanisms that are completely reversible and effective in ridding the cell of excessive FA, provided acceptors such as albumin are available. In a very detailed physical–chemical model, it has been shown that rate and equilibrium constants derived from model system data can account quantitatively for uptake of FA into the liver and provide accurate predictions of intracellular pool sizes (28).

Nevertheless, data derived from model systems may not be convincing to all investigators, especially in light of evidence from several laboratories suggesting that proteins either control or influence the overall "uptake" of FA into cells. These different points of view regarding FA transport have recently been debated in a series of papers (33, 55, 56), and it is not the scope of this review to present all the detailed arguments. Briefly, some of the key arguments for transporters are based on *i*) the saturation kinetics of FA uptake into cells, *ii*) the alteration of FA uptake by modification of proteins, and *iii*) the enhancement of FA uptake by expression of proteins such as the "FA transport protein" (FATP) (57). Counter arguments can be made to all of these points. Observation of saturation kinetics is not sufficient evidence for protein transport, and may reflect partitioning of FA between membranes and albumin, as discussed above and in reference 33. Experiments that modify proteins and show inhibition of FA uptake may interfere with enzymes involved in lipid metabolism or may alter the membrane structure so as to change the partitioning of FA (33). Expression of FATP can increase internalization of FA in 3T3-L1 cells, but the mechanism of its action has not been established, and there is some evidence that the protein works in concert with the enzyme fatty acyl CoA synthetase (57).

Based on the available biophysics data, what step in transport is a protein likely to catalyze? Most investigators have hypothesized that a protein is needed to move the FA across the bilayer, while others have suggested a consortium of proteins working together on all three steps of transport (58, 59). Some investigators have suggested that FA diffuse passively into cells under basal conditions and that transporters increase the uptake of FA above this basal level (57), while others have suggested that transporters work at low concentrations of FA, and that at higher levels of FA, the FA to diffuse into the cell (18). We will conclude with new studies that test both the protein and the passive diffusion models.

Reconstitution studies

One of the most critical tests of a putative transport protein is to remove the protein from the complex cell environment, where many factors can influence transport (any of the three fundamental steps). The purified protein is then reconstituted into phospholipid vesicles to test its possible role in transport of a specific molecule. The only putative FA transporter that has been purified and reconstituted into model membranes is the mitochondrial uncoupling protein (UCP1) found in brown adipose tissue (60, 61). The model for the function of this protein (61) is consistent with the physical properties of FA discussed above. UCP-mediated uncoupling was known to be activated by FA, and the role of the protein was thought to be in H^+ or OH^- transport. To clarify its precise role, the protein was reconstituted into LUV comprised of phospholipids (predominantly soy PC and smaller amounts of cardiolipin and phosphatidic acid) and containing a pH-sensitive fluorophore in the internal aqueous volume (61). Upon addition of FA to the suspension of liposomes (LUV) with or without protein, rapid acidification of the internal buffer was observed (**Fig. 7**). With protein present, the FA activated H^+ efflux from the proteoliposomes. The strongly acidic lipophilic molecule, undecanesulfonate, did not flip-flop across the liposomes (Fig. 7) but was a competitive inhibitor of FA-induced efflux from the proteoliposomes. In the model based on these results (**Fig. 8**), FA diffuse across the membrane in their uncharged form, including H^+ transport, and UCP acts as a pure anion transporter to allow FA to act as cycling protonophores. The full cycle in the Hill diagram of Fig. 3, which is not normally achieved with the flip-flop mechanism, is thereby achieved. Binding of the anionic form of the FA 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 FA (20, 21, 62). It is interesting to note that, in this model FA do arrive in the mitochondrion, as predicted by the physical chemical properties of FA; although they cannot be activated to enter the FA oxidation cycle, they have an unexpected important function in thermogenesis. The complex acylcarnitine transport pathway in mitochondria exists not because FA are unable to enter the mitochondrial matrix but to channel the FA into the oxidation pathway (63).

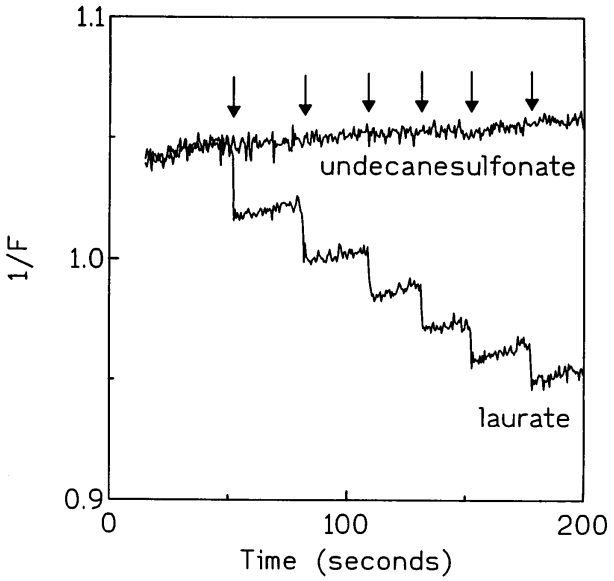

Fig. 7. Flip-flop of lauric acid, but not undecanesulfonic acid, across the LUV membranes. The inverse fluorescence (1/F) is plotted versus time in LUV lacking UCP and containing an internal probe for the detection of H^+ transport. A decrease in 1/F indicates protonation of the fluorescent probe and, hence, delivery of protons across the bilayer membrane. Arrows indicate additions of 25 μm sodium laurate (laurate) or 50 μm sodium undecanesulfonate (undecanesulfonate). (Reproduced with permission from *J. Biol. Chem.* 1996. **271:** 2615–2620.)

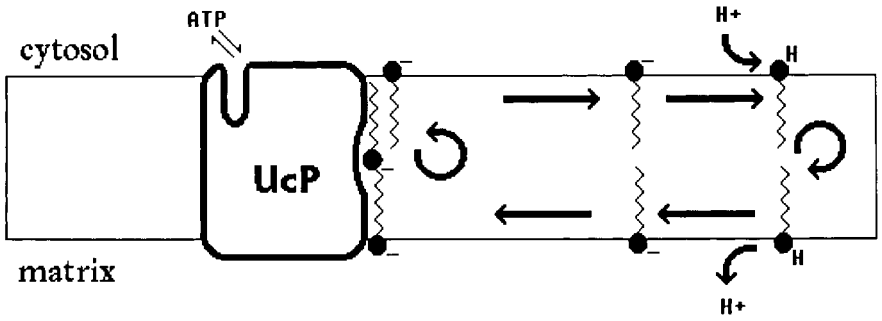

Fig. 8. Proposed protonphoretic mechanism of uncoupling protein. FA bind to the cytosolic leaflet of the inner mitochondrial membrane and cross the membrane by the flip-flop mechanism, delivering H^+ to the matrix. Cyclical flow is achieved by UCP-mediated transport of the anion back to the cytosolic side. This catalyzed protonphoretic cycle dissipates redox energy and produces heat. (Reproduced with permission from *J. Biol. Chem.* 1996. **271:** 2615–2620.)

pH changes in living cells

Passive diffusion of FA into cells is not a new proposal (e.g., 8, 28, 35). However, the supportive data have been generally indirect and apparently unconvincing to many investigators. As in the case of studies that support protein-mediated transport, the transmembrane step has not been studied in isolation from the other steps of transport. To determine whether FA cross the plasma membrane of a cell by the flip-flop mechanism, we have studied intracellular pH effects in cells by FA and analogues of FA. Our hypothesis was that inward movement of FA by diffusion should result in a pH decrease in the cytosolic compartment of the cell. Provided the amount of FA entering is large enough to overcome the buffer capacity of the cell and the protons are not quickly pumped out of the cell, a pH-sensitive fluorophore should record a pH drop, indicating the arrival of FA at the inner leaflet of the plasma membrane. Thus, the approach measured the adsorption and flip-flop steps but not the desorption step. (The adsorption step in cells may be slow compared to vesicles and we cannot assume that flip-flop is the rate-limiting step.) To assess whether any observed pH effect is directly related to FA, albumin or FABP was used to extract the FA from the cell.

Measurements of intracellular pH in adipocytes (**Fig. 9**) supported the mechanism of flip-flop for entry and exit of FA. Upon addition of oleic acid (as aqueous K^+oleate) to a suspension of adipocytes, there was a fast decrease in the intracellular pH ($t_{1/2} < 1$ min) followed by a slower decrease. After the maximal decrease, there was a slow return to the basal pH. BSA caused a rapid alkalinization, suggesting the pH effect is attributed to FA and possibly to their flip-flop. The mechanism by which FA enter the adipocyte was further investigated by using two analogues of FA that should not bind to a putative transport protein and are not metabolized, a FA dimer comprised of two FA cross-linked in the middle of the chain, and an alkyl amine (63). The FA dimer caused rapid and prolonged acidification that was not reversed by albumin. A 14-carbon chain amine, which could flip-flop in its neutral form and then attract protons because it has a net positive charge in the membrane interface, caused an immediate pH increase in the cell. This pH change was also not affected by adding BSA to the buffer. These data show the observed transport is consistent with predictions of the flip-flop model (except for the slower kinetics in the cells) and is not likely to be protein-mediated. The experimental protocol measured the combined steps of adsorption and flip-flop (or desorption and flip-flop when albumin is added), and it is not clear which transport step is slower.

The same experiments with pancreatic β-cells gave similar results for both FA and the FA analogues (64). More recently, digital-imaging microscopy has demonstrated pH changes in single β-cells and adipocytes that are also consistent with passive diffusion across the plasma membrane (F. Caserta, J. A. Hamilton, and B. Corkey, unpublished results). Other investigators have shown that lowering the intracellular pH of adipocytes prior to addition of FA resulted in a decreased amount of FA entering the cell, as predicted by the flip-flop model (65). Although the manipulation of intracellular pH could have some unpredicted effects, their study simulated basal physiological conditions by using low FA/albumin ratios to deliver the FA to cells. In addition, experiments with hepatocytes in which both extracellular and intracellular pH were manipulated gave results consistent with the flip-flop model, although this was not the

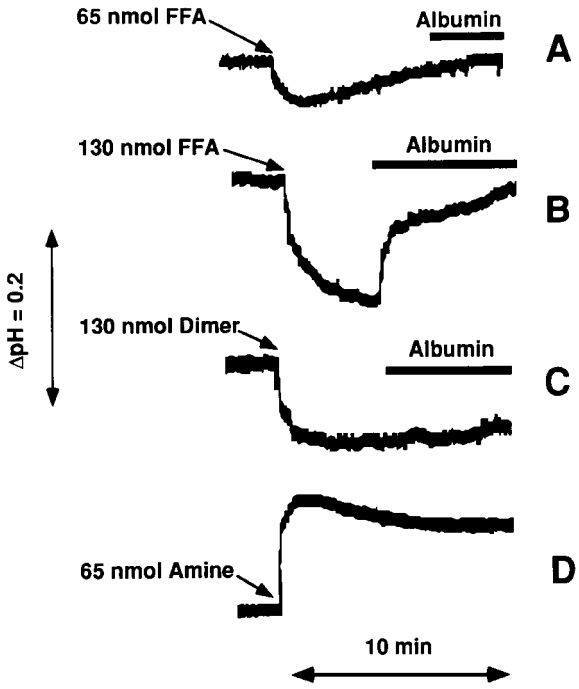

Fig. 9. The time course of change in pH_i after addition of FA to suspensions of isolated rat adipocytes. Rat adipocytes (0.6 mg/ml) loaded with the pH indicator BCECF were suspended in 1.3 ml of Krebs buffer containing no albumin (pH 7.4) at 30°C. Traces A and B show the acidification caused by addition of 65 or 130 nmol of oleic acid as the K^+ salt. The ability of BSA to reverse the effect of FFA addition is compared at different times after addition of FFA (traces A and B). Trace C shows acidification by 130 nmol of a FA dimer. Trace D shows alkalinization by 65 nmol of tetradecylamine. No reversal of the pH_i change occurred upon addition of BSA in traces of C or D. The vertical bar represents a change in pH_i of 0.2 pH unit. (Reproduced with permission from Civelek, V. N., J. A. Hamilton, K. Tornheim, K. L. Kelly, and B. Corkey. 1996. Intracellular pH in adipocytes: effects of free fatty acid diffusion across the plasma membrane, lipolytic agonists, and insulin. *Proc. Natl. Acad. Sci. USA.* **93:** 10139–10144. Copyright 1996. National Academy of Sciences USA.)

interpretation presented by the authors (66). Uptake of cholic acid into liver cells is associated with intracellular acidification (67), which can be explained by passive diffusion of the uncharged form of the bile acid.

CONCLUSIONS

To understand the roles of FA in normal physiology as well as in pathologies such as obesity, diabetes, ischemia, stroke, and inherited metabolic disorders, fundamental properties of FA must be studied rigorously. Knowledge of these properties should be used to design and interpret experiments with more complex systems. The physical chemistry studies of typical dietary FA depict molecules that are dynamic in their environments, not molecules that are static and difficult to move from place to place. This model of FA transport predicts that, in general, FA metabolism regulates transport of FA rather than the reverse. Protein-facilitated transport of acyl carnitine in the mitochondrial membrane is a key point of regulation of FA metabolism.

We hypothesize that passive diffusion through the lipid bilayer is the central mechanism of transport of FA into and out of cells; this mechanism guarantees that FA are supplied to cells and that excess FA can be removed. Specifically, we argue that *i*) the ionization properties of FA in membranes disprove the hypothesis that an anion transporter is required to supply a cell with FA; *ii*) the very rapid rate of flip-flop of FA across model membranes suggests that a pathway through the lipid bilayer in the membrane would short-circuit a protein-mediated pathway; *iii*) the fast rates of spontaneous desorption of typical dietary FA from a phospholipid interface ($t_{1/2} < 1$ sec) may not be rate-limiting for FA metabolism, whereas the slow desorption of VLCFA might be enhanced by a protein; and *iv*) and hypothesis of protein-mediated transport must address the requirement for bi-directional transport across the plasma membrane.

Although our hypothesis is grounded in lipid–lipid interactions, proteins likely play important roles in FA transport. The Na^+/H^+ antiporter may act in response to the movement of H^+ by FA flip-flop and may influence diffusion by local pH changes that change the interfacial ionization of the FA. A membrane protein could influence FA transport by binding FA directly, enhancing partitioning into the membrane, or by playing a role in FA metabolism, for example sequestering FA to a membrane-bound enzyme. If a membrane protein binds FA with high affinity, the rate of release of the FA from the protein will affect the kinetics of transport, possibly slowing it. Elucidating the properties of such proteins in simple model systems where many of the variables can be controlled will help clarify their precise role in transport.

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