J. Membrane Biol. 176, 101–109 (2000)
DOI: 10.1007/s00232001080

The Journal of
Membrane
Biology

Topical Review

Thermodynamics of Fatty Acid Transfer

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Received: 18 January 2000/Revised: 17 April 2000

Abstract. There is continuing controversy about the mechanism for transfer of fatty acids (FA) between plasma and the interior of cells and *vice versa.* One view is that this is a spontaneous process. The generally accepted view is that each step of the process is facilitated by a specialized protein. Whether uptake is spontaneous or facilitated, the components of the uptake system, e.g., albumin, water, FA, plasma membrane, and putative transport proteins of the plasma membrane, must behave according to the rules of the physical chemistry of the system. We review these features to illustrate the constraints they impose on the design of experiments to adduce the mechanism of uptake. Analysis of the literature in the context of the physical chemistry of the uptake system indicates that arguments for a facilitated mechanism of uptake for FA are not supported by any data extant. By contrast, comparison of the rates for individual steps of the pathway traversed by FA moving from albumin to the inside of a cell (or vesicles of a model system) with rates of uptake of FA of tissues in the steady state shows that the rates of the former are sufficient to account for the rate of the latter.

Key words:

Reactions (1) to (4) depict the pathway by which long chain FAs transfer between plasma and the interior of cells. The subscripts out and in refer to the outer and inner halves of the plasma membrane; and the subscript cell denotes that the hydrated FA is inside the cell. The reactions are written as if each reaction proceeds spontaneously.

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$$\text{albumin-FA} + H_2O \leftrightarrow \text{albumin} + \text{FA-}H_2O \quad (1)$$

$$\text{FA-}H_2O + \text{plasma membrane}_{out} \leftrightarrow H_2O + \text{plasma membrane-FA}_{out} \quad (2)$$

$$\text{plasma membrane-FA}_{out} \leftrightarrow \text{plasma membrane-FA}_{in} \quad (3)$$

$$\text{plasma membrane-FA}_{in} \leftrightarrow \text{plasma membrane} + \text{FA-}H_2O_{cell} \quad (4)$$

There is no evidence to the contrary. The generally accepted view, however, is that the energy barriers in (1) to (4) are so high that each is catalyzed by a separate, specialized protein, which is needed to account for observed rates of uptake of FAs into cells [1–4, 9, 14, 20, 46, 48, 64, 71, 72, 79, 82]. Several putative FA transport/binding proteins have been isolated [30, 37, 53, 66, 74, 80, 81]. The data relating to these and the idea of a protein-facilitated mechanism for uptake of FAs by cells, as opposed to a chemically driven, spontaneous mechanism of uptake, have been reviewed on numerous occasions in the recent past [5, 6, 12, 32, 35, 49, 58, 65, 86]. We see no need for another review of these data. On the other hand, review of the literature reveals flaws in experiments designed to adduce evidence for the mechanism by which FA enter cells and that data from these experiments have been misinterpreted. Both problems reflect the failure to consider the physical chemical properties of the system of interest: a water-insoluble ligand (FA) interacting with water, a soluble protein (albumin), and a lipid bilayer (plasma membrane). We review here the physical chemical implications of (1) to (4) that are independent of whether the mechanism of uptake is spontaneous or facilitated. We use a few simple models in which it is easy to see the thermodynamics and kinetic behavior of FA moving between components. The mod-

els are constructed with increasing complexity of elements and range from static models in which equilibrium states will be achieved to a dynamic model with metabolism of FA, which is expected to reach the steady state rate of uptake = rate of metabolism rather than an overall equilibrium state. The analysis emphasizes how thermodynamic and kinetic realities impose constraints on the design of experiments for elucidating the mechanism of uptake of FA into cells.

Thermodynamics

Model 1: The Distribution of FA Between Albumin and Empty Lipid Vesicles

We describe first how FAs distribute at equilibrium between albumin in (1) and the lipid region of a membrane (in (2)). Modeling the system in this way is fully justified because, independent of whether FA enter cells by a passive or facilitated mechanism, there is overwhelming evidence for the ready solubility of fatty acids in phospholipid membranes.

When complexes of albumin-FA are dissolved in water, FA dissociates from albumin to satisfy the thermodynamic relationship in (5).

$$K_d^{alb} = [\text{FA}]_{water}\,[\text{albumin}]/[\text{albumin-FA}] \quad (5)$$

If some amount of lipid-containing membrane, e.g., pure lipid bilayer or the lipid bilayer region of a membrane, is added to the system, FA in water rapidly enters the membrane phase. This event drives dissociation of FA from binding sites on albumin. FA moves between the albumin and lipid compartments until the equilibrium conditions of Eq. (6) are reached, which applies because FA in membrane and albumin phases is at

$$(K_d^{alb})\,([\text{albumin-FA}]/[\text{albumin}] = (K_d^{memb})\,([\text{membrane-FA}]/[\text{membrane}] \quad (6)$$

equilibrium with FA in a common aqueous phase. FA also will shift in the reverse direction if albumin is added. FAs do not need to run down a thermodynamic gradient in order to shift from albumin to lipid phase. The chemical potential difference between FA in different compartments only sets the equilibrium concentrations that satisfy the condition of (6).

Equation (5) suggests that $[\text{FA}]_{water}$ can be calculated for the uptake system, which is a common basis for analyzing data for uptake of FA into cells [*c.f.* 3, 10, 12, 48, 64 for representative treatments]. However, albumin contains several sites for binding FA, with variable affinity for FA [17, 36]. Therefore, a tabulated value for K_d^{alb} will apply only at the instant of mixing and then vary with time as FAs move from albumin to membrane.

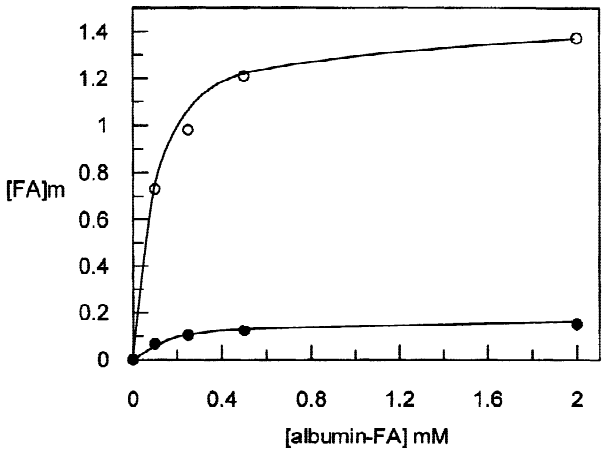

Fig. 1. Equilibrium value of $[\text{FA}]^{memb}$ as a function of [albumin-FA] at moles FA/moles albumin = 0.5 (●) and = 4 (○). The concentration of membrane lipids was constant. [FA]m is moles FA/moles phospholipid. For details *see* [56].

Because of a large difference in rate constants for the forward reactions of (1), which is slow, and (2) which is fast [24], it cannot be assumed that $[\text{FA}]_{water}$ is at its equilibrium value even at the instant of mixing. Values of K_d^{alb}, $[\text{FA}]_{water}$, [albumin], and [albumin-FA] in a mixture of albumin-FA complexes and membranes in water cannot be calculated from tabulated values of K_d^{alb}, except at the equilibrium position in (6). Another consequence of the chemistry of the system is that membrane lipid will appear to become saturated with FA at a finite value for $[\text{FA}]_{memb}$ as more albumin-FA complex (at a fixed, initial mole ratio FA/albumin) is added to fixed amount of lipid bilayer. This simple chemical property is demonstrated in Fig. 1, which shows the relationship between $[\text{FA}]_{memb}$ and [albumin-FA] for different initial ratios moles FA/moles albumin. The physical chemistry of the system has behavior that could be interpreted as "biological" [56]. The value of $[\text{FA}]_{memb}$ that "saturates" the lipid phase depends on the nominal value of the mole ratio FA/albumin [56]. The lipid bilayer phase hence does not truly become saturated with FA; and $1/y$ intercepts in a double reciprocal plot of the data in Fig. 1 are apparent values for saturating concentrations of FA. So despite apparent "saturation" of binding in plots like Fig. 1, one species of FA does not compete with another for binding to the membrane [23]. On the other hand, a pure lipid bilayer contains a maximum number of "sites." Pure lipid phases can be saturated by a number of hydrophobic compounds at high concentrations of the latter [8, 29, 41, 50, 62, 63].

The concentration of FA that appears to saturate the membrane in Fig. 1, for mole ratio FA/albumin = 0.5 (within the physiologic range), is about 2 mole percent. This value is high in the context of a nontoxic, physiologic concentration [41–44]. It is also high compared

with concentrations achieved in vivo. Under physiologic conditions for concentrations of plasma membrane and albumin, for example, with a nominal ratio of 0.5 moles FA/mole albumin the concentration of palmitate in plasma membrane lipids is 0.06 mole percent [56]. At a nominal value of 4 moles FA/mole albumin (above physiologic range), the concentration of palmitate rises to only 1.4 mole percent of plasma membrane lipid [56]. The high avidity of albumin for FA provides a vehicle for bulk phase transport and a buffer system for FA in blood [22]. The avidity of albumin for FA is so great that concentrations of FA in membrane lipids are kept at low levels under physiologic conditions [22, 56].

Theory predicts that K_d^{memb} will be impacted by chemical and physical changes within the lipid phase [25, 26, 84], which is substantiated by direct experimental observation [51, 57, 76]. Experimental design for studies of the uptake problem must consider, therefore, the potential effects of experimental variables on K_d^{memb}. For example, phloretin is used to wash cells in FA uptake experiments, on the assumption that it inhibits facilitated transport [*c.f.* 1–4, 9, 14, 20, 46, 48, 64, 71, 72, 79, 82]. Phloretin, however, dehydrates the lipid-water interface and expands lateral separation of adjacent phospholipids [39]. Phloretin also inhibits spontaneous transfer of FA across pure phospholipid bilayers [33, 34].

Model 2: The Distribution of FAs Between Albumin and Lipid Vesicles Containing a Soluble FA Sink of Finite Size

Cells contain interior compartments to which FAs bind, including soluble FA binding proteins [60], intracellular membranes and lipid droplets in cells like adipocytes. This situation can be modeled by trapping a FA inside a vesicle by having it bind to a protein. In this setting, some of the FA bound initially to albumin will become bound to the FA-trap inside the vesicle, as FA equilibrates across the outer and inner halves of the bilayer and becomes hydrated (reaction (4)) inside the vesicle. More FA will shift from albumin to the total vesicular compartment in the presence of a soluble FA-trap *vs.* an empty vesicle. The extra amount of FA moving from albumin to the inside of the vesicle will affect the equilibrium distribution of FA between albumin and lipid because, as noted already, K_d^{alb} at equilibrium changes as the mole ratio FA/albumin declines. The extent to which K_d^{alb} is perturbed at equilibrium for model 2 depends on the size of the trap, which includes its avidity for FA. In addition to perturbing the value of K_d^{alb} at equilibrium, a FA-trap, e.g., intracellular FA binding protein, adds the property of competition because, in contrast to the lipid phase, the second protein phase has a far more limited binding capacity for FA. But we note the site at which competition occurs is independent of the events of uptake *per se.*

Model 3: The Distribution of FAs Between Albumin and Lipid Vesicles Containing a Membrane-Bound FA Sink of Finite Size

This adds considerable complexity to the system. A membrane bound FA trap has effects on K_d^{alb} and the equilibrium position of (6) similar to those for a soluble trap in the interior of a vesicle. Additionally, however, if the protein is integral to the membrane, it will perturb the packing of lipids of the membrane bilayer and thereby alter K_d^{memb}. An integral membrane protein creates defect structures at the protein-lipid interface within the hydrophobic interior of the bilayer, for example [38]. Depending on the protein, its concentration in the membrane, and the lipid composition of the bilayer, an integral membrane protein can influence packing between polymethylene chains of phospholipids not at the protein interface. One cannot assume, therefore, that an integral membrane protein that binds FA in vitro has this singular effect in a membrane. The validity of this assumption has to be established experimentally. Without knowing that K_d^{memb} is constant for variable experimental conditions, data from such experiments, e.g., after stable overexpression of candidate proteins in cell lines [*c.f.* 8], cannot be interpreted.

Determining whether or not a putative FA binding protein (in the plane of a membrane) binds FA at a specific site or changes K_d^{memb} is another difficult experimental issue. It does not reduce to measuring the difference between [FA] in the membrane component of the system with and without the protein of interest. Extra FA measured in the former instance could be solvated by bulk lipid phase or could be bound at a specific binding site on a putative binding/transport protein. The only certain way to determine the location of the extra FA in this experiment is to utilize integral membrane protein molecules with optical signals that are activated or quenched when ligands bind to specific sites. This has yet to be accomplished. Using fluorescent FAs is problematic because structural differences between parent and fluorescent-labeled FA influence binding to albumin, solubility in the bilayer, and so on.

Experimenters have tried to get around the problem in simple ways. Careful reading of the literature leads to the inescapable conclusion that experimental designs to this end are biased [1–4, 9–11, 14, 20, 46, 48, 64, 71, 72, 74, 79, 82]. Experiments leading to the conclusion that uptake is facilitated already assume that FA not bound at the ligand binding site of a protein intrinsic to the uptake mechanism is nonspecifically bound to membranes or cells and has no relationship to the uptake system. Further assumptions are made. FA not bound to a specific ligand site on a protein should be removed from the system prior to analysis of the amount of FA taken up by the system. And the so-called “nonspecifically bound FA” can be removed quantitatively and selectively by

washing cells or membranes with albumin [*c.f.* 3, 10, 11, 48, 64, 72 for representative experimental designs].

The purpose of the uptake experiments is to adduce information on the mechanism of uptake of FA. Yet the design of the relevant experiments purposely discards information relating to a nonfacilitated mechanism. These data are excluded from final observations and from consideration. This logical flaw is compounded by experimental uncertainty about the amount of FA actually taken up by a system because it is assumed that "nonspecific fraction of FA" is removed selectively by washing with albumin. However, albumin added to model 3, after FA has equilibrated between components, removes FA from all components to which FA is not bound covalently. It can be argued that more FA can be extracted by albumin from compartments with relatively poor *vs.* high affinity for FA; but there are no data to establish the relative affinities of putative uptake components for FA, except as reviewed already for albumin and lipid membranes in model 1. Washing with albumin thus not only removes information from the system. It also perturbs information about interactions between FA and putative transport and/or binding proteins.

There is still another level of complexity to the interaction of FA with the membrane-bound FA-trap in model 3. FA is hydrated by the water phase (on the outside of the vesicles) and solvated by the lipids within the membrane. Which pool of FA interacts with the membrane-bound trap to form the protein-FA complex? The simple answer is with both, but not equally so. There is a compelling thermodynamic basis for concluding that the pool of FA within the membrane is the proximate pool interacting with an integral-membrane, FA-trap.

Reaction (7) is the rate for binding of FA in water and (8) for FA within the membrane to a putative membrane binding protein, which could bind FA only and/or catalyze (3). Because of the difference in the concentrations of components in the water and membrane phases,

$$v_{water} = k_7\,[\mathrm{FA}]_{water}\,[\text{membrane protein}]_{water} \qquad (7)$$

$$v_{memb} = k_8\,[\mathrm{FA}]_{memb}\,[\text{membrane protein}]_{memb} \qquad (8)$$

v_{water}/v_{memb} can be on the order of $>10^{-10}$. Besides this advantage for binding via the lipid bilayer, transfer of FA from water to membrane decreases its entropy of motion. This makes the free energy change smaller for (8) than for (7) because binding in the former instance is entropically less costly. Therefore, $k_8 > k_7$. There is little work on the interaction of integral membrane enzymes with substrates that dissolve within the lipids of the bilayer. There are, however, relevant data for microsomal acyl-CoA ligase, for which FA is the membrane-soluble ligand. $[\mathrm{FA}]_{memb}$ not the aqueous phase pool of substrate interacts with this enzyme to form the enzyme-substrate complex [54, 55].

Since FA dissolves readily in bilayers, (3) is the only membrane event in the uptake of FA for which the spontaneous event might be too slow for physiologic needs. Assume, therefore, that the membrane trap in model 3 catalyzes (3). Important consequences follow from whether (7) or (8) describe the step leading to the enzyme-substrate complex for a catalyzed (3). As mentioned already, interpretations of binding data or rate data are made in terms of aqueous phase concentrations of FA [*c.f.* 3, 10, 12, 48, 64 for representative experiments]. This is not necessarily incorrect because $[\mathrm{FA}]_{water}$ is a dependent variable that can be calculated from (6). None of the work on the uptake problem that we can find calculates $[\mathrm{FA}]_{water}$ on the basis of (6), however. $[\mathrm{FA}]_{water}$ is calculated instead from K_d^{alb} for the ratios moles FA/moles albumin used in an experiment and an algorithm that accounts for binding to multiple sites at any given ratio of moles FA/moles albumin. The discussion of Model 1 illustrates that this is an improper use of data because it is based on assumptions that are not likely to prove valid. Simple calculations indicate that differences between true and calculated values of $[\mathrm{FA}]_{water}$ could exceed 2-fold, depending on the conditions of an experiment; so this is not a trivial point. Additionally, the real value of $[\mathrm{FA}]_{water}$ changes continuously over the time course of an experiment. These uncertainties can be avoided by using very high concentrations of albumin-FA complexes in assay systems. Under this condition, only a small fraction of FA shifts from albumin to the membrane, which keeps K_d^{alb} constant at its nominal, initial value, but does not assure that $[\mathrm{FA}]_{water}$ is at its equilibrium value, over the course of an experiment.

The lack of physical meaning in calculations of $[\mathrm{FA}]_{water}$ [see 3, 10, 12, 48, 64 for representative experiments] is further illustrated by implications from the data in Fig. 1. It is easy to show with these data that a lipid bilayer can have 2 or more equilibrium concentrations of FA for a given value of $[\mathrm{FA}]_{water}$, in the absence of changes of any other thermodynamic variable. This cannot be; so the calculation of $[\mathrm{FA}]_{water}$ from (1) must not apply to the system of albumin-FA plus membrane.

Model 4: The Distribution of FAs Between Albumin and Lipid Vesicles Containing a Soluble FA Sink of Infinite Size

The FA trap in this iteration of the model is a metabolic system. This feature causes the transfer of FA from albumin to the model cell to run down a thermodynamic gradient. Whereas the chemical potential of FA is higher in water, or membrane, or bound to an intravesicular trap, as compared with binding to albumin, which causes the three prior models to run until an equilibrium distri-

bution of FA occurs for all components, the metabolic trap of infinite capacity draws all FAs into it. Intermediate reactions in the pathway from albumin to entry into the metabolic trap might or might not be at their equilibrium positions, in this model; but FA does not reach an equilibrium state within the metabolic trap. The lower free energy for the products of metabolism *vs.* FA bound to albumin pulls the overall reaction to essential completion. Whether none, any, or all the intermediate reactions in the pathway are at their equilibrium positions depends on their rates relative to each other and the rate of metabolism. If the rates of the intermediate reactions are fast relative to the rate of metabolism, the intermediate reactions will be at equilibrium. If the rate of metabolism is fast relative to intermediate reactions, intermediate reactions may achieve steady-state concentrations of FA different from the equilibrium positions of the system. Or some of the intermediate reactions will be at equilibrium and others at steady state. If (1) and (2) are not at equilibrium during transfer of FA in model 4, $[FA]_{water}$ cannot be calculated from any of the observables.

Kinetic Considerations

Model 1

Abumrad and colleagues [1] have stated that the problem of determining the mechanism for the uptake of fatty acids is to analyze rate data in the context of the Michaelis-Menten formalism. "Determination of the initial rate of uptake of a substance as a function of its external concentration can distinguish simple diffusion (a linear relationship) from transport (a saturable process)." Most workers in the field have followed this lead. Thermodynamic analysis of models 1 to 3 shows, however, that a simple chemical process can reach limiting values (become saturated) at finite concentrations of the experimental variable. Moreover, the Michaelis-Menten formalism does not apply to analysis of any of the models discussed.

Consider the kinetic behavior of model 1, in which there is no transfer of FA from outer to inner halves of the bilayer. If all FA is present initially on albumin and lipids are added, the rate of decline of FA in the albumin compartment (or appearance of FA in the lipid compartment) is given by (10), in which the superscript *om* means outer half of bilayer.

$$-d[FA]_{alb}/dt = k_1\{[FA]_0^{alb} - [FA]_t^{om}\} - k_{-2}[FA]_t^{om} \quad (10)$$

The rate constants k_1 and k_{-2}, respectively, are for hydration of FA bound to albumin or membrane. The rates for binding of FA to albumin or membrane are not considered because well-known binding constants indicate that they are orders of magnitude larger than those for hydration. The progress curve for the rate equation in (10) depicts the approach of the system to its equilibrium position. It will not be zero order. Nevertheless, Michaelis-Menten assumptions have been made in order to estimate initial rates, extrapolated from nonlinear rates or even from data at a single time point [*see* 3, 10, 11, 48, 64, 72 for representative examples]. But the 'initial rate" of uptake of FA into any membrane in (10), for an experiment in which FA is added as albumin-FA complexes, is $k_1[FA]_0^{alb}$. The "initial rate" in a typical FA-uptake experiment, assuming that this rate can be estimated with a reasonable accuracy, contains information only about the albumin compartment. Equation (10) also shows that it is trivial to demonstrate increasing rates of uptake under the condition that either k_1 or $[FA]_{alb}$ increase. Modulating the rate of uptake by adding albumin-FA complexes with varying ratios of moles FA/moles albumin thus yields a trivial result.

If the rate constant for transbilayer movement of FA is not zero, then (11) describes the progress curve for transfer of FA from albumin to membrane, where k_3 is the rate constant for reaction (3), which we assume is the same for forward and reverse directions.

$$-d\{FA]_{alb}/dt = k_1\{[FA]_0^{alb} - [FA]_t^{om} - [FA]_t^{im}\} - k_{-2}[FA]_t^{om} + k_3[FA]_t^{om} - k_3[FA]_t^{im} \quad (11)$$

The progress curve for movement of FA from albumin to membrane in (11) still describes the time course for approach to equilibrium and is not zero order. The initial rate of transfer remains $k_1[FA]_0^{alb}$; and (11) applies whether reaction (3) occurs spontaneously or is a catalyzed event. If $k_1 >> k_3$ and $k_{-2} >> k_3$, then the distribution of FA between albumin and the outer half of the bilayer will reach equilibrium before FA equilibrates between halves of the bilayer. Rate data in this case will be best fitted by 2 exponential terms. The first contains information about reactions (1) and (2) while the second exponential contains information about reaction (3). But if $k_3 >> k_1$ and k_{-2}, the progress to equilibrium will be a single exponential and provide no information on the rate of reaction (3). The same is true in the case that $k_3 = k_1$ and $k_3 = k_{-2}$. This simple analysis of a very simple system illustrates, therefore, the experimental difficulty of extracting meaningful rate data about single steps that are a part of a sequential pathway.

Models 2 and 3

Putting an FA trap inside a vesicle (model 2) or inside a membrane (model 3), or both, does not change the fundamental applicability of (10) and (11) as opposed to a rate expression that can be analyzed according to "Mi-

chaelis Menten kinetics." Additional rate terms have to be added to (11), but these terms will not alter the physical significance of the so-called initial rate of uptake. This still equals $k_1[FA]_0^{alb}$. Whether or not uptake is facilitated, FAs move in models 2 and 3 from albumin to other compartments until an equilibrium distribution is reached, and the time-dependence of this process again is not zero order. Not surprisingly, the extent of uptake of FA can be driven by increasing the capacity of FA traps [10, 11, 48, 64], but this observation provides no insight into mechanism.

Model 4

The kinetic pattern of model 4 is different from the other models because there are 2 separate rate processes in 4. The first rate process is for loading of nonmetabolic compartments with FA, to reach either equilibrium or steady-state values for [FA] as discussed already. The second rate process is for the metabolism of FA. If progress curves for disappearance of FA from albumin are followed, there will be a pre-steady-state phase corresponding to the loading of the nonmetabolic compartments, followed by a steady-state rate that equals the rate of metabolism of FA. The shape of the progress curve during the pre steady state can be convex, depending on the relative rates of events in the pre steady state, loading phase of an experiment and the rate of metabolism in the steady state. If the appearance of free FA inside the vesicle of model 4 is the observable followed, the progress curve for uptake will look like those for the other models because [FA] in nonmetabolic compartments approaches equilibrium or steady-state values. The appearance of FA inside the vesicle is the least useful observable of the system because most FA will be in the form of metabolic products; and the fraction oxidized to CO_2, which could be large, is lost. Independently of the observable followed, whether rates of processes for loading and metabolism can be separated from each other depends on their relative rates. The events of uptake *per se*, e.g., the loading phase, can be studied only if the pre steady state rate is far greater than the steady state rate. It is not known whether this condition exists for any real cell under the conditions that have been employed to study the "uptake" process. It has been shown for perfused liver that the metabolic rate is relatively large as compared with the loading rate [22]. Total [FA] in the cells of perfused liver cell is constant with time after a steady state rate of uptake is achieved; but this concentration is less than required for an equilibrium distribution of FA between albumin and non-metabolic compartments of the liver cell [22]. These specific results mean that the rate(s) of events of the uptake process cannot be studied in perfused liver because the rate(s) of these events are obscured by contributions from the metabolic rate. In other words, it cannot be assumed that the true events of uptake, i.e., the nonmetabolic events, can be measured directly in a given metabolic system. This possibility must be established experimentally.

There is a relationship between the extent to which the nonmetabolic components of model 4 are loaded and the steady state rate of uptake. (12) is the rate equation for metabolism of FA via acyl-CoA ligase (first metabolic step for esterification or oxidation) at fixed [ATP] and [HS-CoA], where [FA] is concentration in the endoplasmic reticulum [22, 54, 56].

$$v = K_M/V_{max}[FA][ligase] \quad (12)$$

[FA] is directly proportional to $[FA]_{memb}$, whether model 4 is at equilibrium or a steady-state condition. This means that rate of uptake in the pre-steady state and the steady state is determined by thermodynamic variables that affect K_d^{alb} or K_d^{memb}, such as temperature [75] and electrochemical gradients [73, 83].

Whether or not equilibrium is achieved for the nonmetabolic components of the uptake system, the pre-steady-state phase will be relatively brief. Care has not been taken to distinguish between the pre-steady-state rate and the steady-state rate of uptake or to be certain that these rates can be separated in an uptake experiment. This failure also confounds interpretation of data in the literature. Thus, examination of the models shows that inhibitors of the metabolism of FA, of the TCA cycle, and of oxygen consumption will appear to inhibit the "uptake" of FA. Conclusions have been made about mechanism of uptake on the basis that steady-state rates of uptake were altered, typically by thiol reagents [1]. Obviously, inhibitors of metabolism that have no relation to the mechanism of uptake will diminish the steady state rate while providing no data on mechanism. The same applies to interpreting experiments in which some manipulation has increased the rate of uptake of FA in the steady state. According to (12) this kind of result will be caused by an increase in [FA], which could be due to an enhanced rate of transfer of FA to the FA metabolizing compartment or to a change in K_d^{memb}, or to an increase in [ligase] or another FA-metabolizing enzyme, which would mean a change in metabolic capacity. All the possibilities that could account for changes in rates of uptake in the steady state, as experimental conditions are varied, must be accounted for explicitly. Otherwise no conclusions can be made about the reaction in the overall path for uptake, i.e., steps (1) to (4) and metabolic steps, that is affected by the variable of interest.

Indirect Evidence on the Mechanism of Uptake of FA into Cells

Figure 1 shows that the criteria that have been used to distinguish between a facilitated or nonfacilitated mecha-

nism for uptake of FA (given explicitly in ref. 1) are not valid. Constraints imposed by the thermodynamics and kinetic behavior of the uptake system for FA indicate that experimental design in the applicable system is more difficult than has been appreciated. It is unlikely, in fact, that any set of experiments with intact cells or tissues can yield definitive information about the mechanism. On the other hand, simple experiments in model systems, when combined with data from cells, can yield insight into mechanism. Model 1, for example, contains all the steps traversed by FA moving from plasma to the inside of a cell. One or all the steps could be slower or faster than the steady-state rate of uptake of FA in cells. If one (or all) of these events were slower (in model 1) than steady-state rates of uptake of FA in cells, then indirect studies would suggest facilitated uptake of FA by cells. Experiments could be contemplated, in this case, to measure the rates of (1) to (4) in a facilitated uptake system constructed from putative proteins of the uptake system and model 1, which generates model 3. If all the steps (1) to (4) were faster in model 1 than steady-state rates of uptake of FA in cells, then results would suggest that uptake was spontaneous or that the cell had a mechanism to slow uptake *vs.* the expected spontaneous rate. The latter is hard to visualize, however, because plasma membranes have bulk lipid phase arranged as the bilayer of model 1.

The data for rates of (1) to (4) in model systems like model 1 can be summarized as follows. (1) is the slowest of reactions (1) to (4) [7, 18, 24, 28, 52, 56, 61, 67, 69, 77]. The rate constant of (1) is sufficiently large, however, to account for the steady state rates of uptake of different FA by perfused liver at physiologic ratios of moles FA/moles albumin [22, 56]. The rate constants for (2) and (4) are several orders of magnitude greater than (1); so neither of these is rate limiting for uptake [7, 18, 24, 28, 52, 56, 61, 67, 69, 77]. We note that $k_2 >>> k_{-2}$ or k_4 [24]. There is controversy, however, about the value of k_3 and its relationship to rates of uptake of FA in cells. Estimates of k_3 from data for naturally occurring FA indicate that k_3 is orders of magnitude larger than required to account for steady-state rates of uptake of FA into cells [18, 24, 44, 55]. Estimates of k_3 from data with anthroyloxyFA analogues have been interpreted to mean that k_3 is too small to account for steady-state rates of uptake by FA by cells [42–45, 70]. The geometry and volume of fluorescent analogues of FA are different from the parent FA, which could impact the value of k_3. There are no measurements of the steady-state rates of uptake into cells of the analogues of FA for which k_3 is reported to be relatively small. Nevertheless, k_3 for anthroyloxyFA in some experiments [70] exceeds k_1 [18, 28, 52, 69]. Contrary to the bias implicit in [1–6, 9–12, 14, 20, 37, 46, 48, 49, 64–66, 71, 72, 74, 79–82] i.e., that transbilayer movement is inherently slow in the absence of catalyst, theoretical considerations show convincingly that rapid transbilayer diffusion is expected for molecules that dissolve in the apolar region of a bilayer [21, 41, 78]. This prediction has been validated for an array of these compounds [8, 13, 15, 16, 19, 27, 40, 47].

In summary, the most that can be said with certainty about the uptake system for FA in cells is that measurements of reactions (1) to (4) support the conclusion that uptake could be a spontaneous process. Each step of (1) to (4) is faster than what is needed to account for measured steady-state rates of uptake of FA into cells. Although these observations do not prove the mechanism of uptake, they make it difficult to imagine why nature would use resources for a purpose not needed.

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