1979, 1982). This shows that the free energy change obtained by extrapolation of the unfolding equilibrium curve to 0 M denaturant represents well the free energy change for the unfolding reaction that actually occurs in the absence of denaturant. The same may hold well for the extrapolated free energy changes obtained for other proteins (Pace, 1975).

Hydrogen isotope exchange has been used extensively as one of the methods to study the fluctuations of the protein molecule. In order for hydrogen atoms located in the interior of the protein molecule to be exchanged with deuterium, the former must be exposed to solvent. Although it is established that hydrogen isotope exchange proceeds through global unfolding in the unfolding transition zone, it is not clear from hydrogen exchange experiments whether global unfolding of the protein molecule occurs as well under native conditions (Woodward & Tüchsen, 1982). However, the results obtained in the present study strongly support the proposal that the C_L fragment molecule undergoes global unfolding even under native conditions.

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Physical–Chemical Model for the Entry of Water-Insoluble Compounds into Cells. Studies of Fatty Acid Uptake by the Liver†

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ABSTRACT: The spontaneous transfer of water-insoluble substances from plasma to the interior of cells would involve a series of steps in which the substance of interest dissociates from albumin in plasma, enters the outer half of the plasma membrane of a cell, crosses the bilayer, and then dissociates from the inner half of the plasma membrane to enter cell cytosol and diffuses to sites of its metabolism. We have examined the behavior of long-chain fatty acids in the uptake process, assuming that none of these steps is facilitated by the cell during the entry of fatty acids into the liver. Comparison of the spontaneous rates for each individual step with rates of uptake of fatty acid by perfused liver leads to the conclusion that the uptake of fatty acids is not limited by kinetic factors but is determined instead by the equilibrium distribution (K_{eq}) of fatty acids between albumin in plasma and the phospholipids of the plasma membrane. This idea was examined further by determining whether there was a relationship between the value for K_{eq} and rates of uptake of a fatty acid and the pattern of kinetics for uptake. The data indicate that there is a linear relationship between K_{eq} and the rate of uptake, that uptake rates can be predicted with a high degree of accuracy from thermodynamic data, and that the pattern of kinetics of uptake is compatible with the idea that the uptake rate is determined by the relative affinity of a fatty acid for albumin and membranes.

Mechanisms for the uptake of water-insoluble substances by tissues have been considered in terms of processes for uptake of water-soluble substances. Thus, most if not all, treatments of the subject are conceptualized as requiring specific mechanisms for the transport into tissues of water-insoluble compounds (Mahadevan & Sauer, 1971, 1974; Samuel et al., 1976; Weisiger et al., 1981; Abumrad et al. 1981, 1984; Stremmel et al., 1985). The problem for the cell in internalizing polar compounds is to short-circuit the high energy barrier to the passage of these substances across the apolar plasma membranes of the cell. By contrast, many biologically important compounds with limited solubility in water are highly soluble in membrane lipids and in fact penetrate lipid bilayers in a

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facile manner (Oldendorf, 1974; Deuticke, 1977; Eibl, 1984). The problem for cellular metabolism of nonpolar compounds probably lies not in internalizing these substances but in keeping concentrations at minimal levels in intracellular membranes. Failure to consider the basic differences in the physical–chemical interactions between membranes and polar compounds vs. the interactions between membranes and nonpolar compounds may have obscured the fundamental processes that control the distribution between organs and rates of uptake into specific organs of substances with limited solubility in water. This seems to be true for the problem of the uptake of long-chain fatty acids by tissues.

Long-chain fatty acids are quantitatively the most important substrates for energy production in cells. There has been considerable interest, therefore, in the mechanism by which these compounds are taken up by cells. The uptake process has been studied by using a variety of preparations from different mammalian sources; yet no consistent proposal for the mechanism of uptake and the nature of the rate-determining step(s) has emerged (Mahadevan & Sauer, 1971, 1974; Samuel et al., 1976; Weisiger et al., 1981; Abumrad et al., 1981, 1984; Stremmel et al., 1985; Spector et al., 1965; DeGrella & Light, 1980a,b; Weisiger, 1984). There is uncertainty as to how fatty acids are transferred from plasma to the plasma membranes of cells. Transfer is believed to occur spontaneously (Spector et al., 1965; DeGrella & Light, 1980,b), for example, or to be facilitated by a specific receptor on the plasma membrane of cells (Mahadevan & Sauer, 1971, 1974; Samuel et al., 1976; Weisiger et al., 1981; Abumrad et al., 1981, 1984; Stremmel et al., 1985). Kinetic data for uptake of fatty acids by cells have been related to the aqueous phase concentration of the complex albumin–fatty acid (Weisiger et al., 1981; Abumrad et al., 1981, 1984), or to the total amount of fatty acids in the system (Mahadevan & Sauer, 1971, 1974; Samuel et al., 1976; DeGrella & Light, 1980a,b), or to the concentration of fatty acids calculated to be in the aqueous phase (Abumrad et al., 1981, 1984). The concentration of the membrane-associated pool of fatty acid is not considered to be related to processes for uptake nor has attention been given to the consequences of the solubility of fatty acids in cellular membranes (Mahadevan & Sauer, 1971, 1974; Samuel et al., 1976; Weisiger et al., 1981; Aburmad et al., 1981, 1984; Stremmel et al., 1985; Spector et al., 1965; DeGrella & Light, 1980a,b; Weisiger, 1985). Interestingly, even the concentrations of fatty acids in the aqueous phase of uptake systems are calculated from values of K_d for the dissociation of albumin–fatty acid complexes in water. These calculations do not take into account the predominant distribution of fatty acids between albumin in the vascular space (or aqueous media) and the lipid bilayers of cell membranes.

In the present work, we propose a model for the cellular uptake of fatty acids that considers the consequences of the partitioning into membranes of hydrophobic metabolites during the process of their uptake by cells. We have tested the proposed model by evaluating rates for individual steps in the uptake process and by examining predictions made from the model about the kinetic behavior for the uptake of palmitate by perfused rat liver. This analysis appears to provide a more reasonable understanding of the processes involved in cellular uptake of fatty acids than is provided by existing schemes. We believe, moreover, that this model may apply generally to the processes involved in the cellular uptake of hydrophobic metabolites.

Materials and Methods

Equilibrium Distribution of Palmitate between Lipid Bilayers and Albumin. The equilibrium distribution of palmitate between unilamellar vesicles of egg phosphatidylcholine and albumin was determined by the method of Backer and Dawidowicz (1979). Bovine serum albumin (0.2 mM) (Sigma, essentially fatty acid free) in 10 mM tris(hydroxymethyl)-aminomethane (Tris), 100 mM KCl, and 1 mM ascorbate, pH 7.4, was mixed with palmitate at a mole ratio of 6:1 (palmitate:albumin ratio). A trace amount of [^{14}C]palmitic acid (Amersham) was included. This solution was diluted with 0.2 mM albumin to obtain various palmitate:albumin ratios. Vesicles were prepared from egg phosphatidylcholine (Sigma, type III-E in hexane) by sonication. They contained 10% (w/w) *N*-palmitoyldihydrolactocerebroside (Sigma). The lipids then were sonicated to obtain a clear suspension that was centrifuged at 100000*g* for 15 min in order to sediment undispersed material. Vesicles and complexes of albumin–palmitate were incubated at 37 °C for 1 h. Following the incubation, a sample of the mixture was transferred to an Eppendorf tube, and 0.3 mg of lectin (RCA 120, Vector Laboratories, Burlingame, CA) was added to it. The mixture was mixed vigorously, kept at 37 °C for 10 min, and centrifuged for 10 min. Aliquots (200 μL) of the supernatant were then counted for [^{14}C]palmitate. To correct for unprecipitated vesicles, vesicles that contained trace amounts of [^{14}C]dipalmitoylphosphatidylcholine (Amersham) and cerebrosides were prepared, incubated with 0.2 mM albumin solution, and precipitated by lectins as described, and 200 μL of supernatant was counted for unprecipitated lipids. The amounts of palmitate bound to albumin (in the supernatant) and to the lipid vesicles (in the pellet) were calculated at each palmitate:albumin ratio, and the equilibrium constant was calculated from the expression

$$K_{eq} = \frac{\text{mol of palmitate (in vesicles)/mol of lipid}}{\text{mol of palmitate (on albumin)/mol of albumin}}$$

Uptake of Palmitate by Perfused Rat Liver. Male Wistar rats (Charles River breeding laboratories) weighing 220 ± 20 g (mean standard error) were anesthetized with methoxyfluoran. Each liver were perfused in situ by using a modification of the technique of Avner et al. (1981). The perfusate was circulated by a peristaltic pump (LKB, Multiperpex 2115). The medium was kept at 38 °C and was equilibrated with a mixture of 95% O_2 and 5% CO_2 at pH 7.4. The portal vein was cannulated with an 18-gauge catheter (Angiocath, Deseret Co.). The liver then was flushed with heparin (500 USP units in 5 mL of saline), and perfusion was started at a slow rate of about 8 mL/min. The inferior vena cava was cannulated with a 16-gauge catheter, and the outflow was connected to the peristaltic pump. The superior vena cava was tied off, and the perfusion rate increased to about 30 mL/min. Perfusions were started by recirculating oxygenated fluorocarbon (oxypherol, Alpha Therapeutic Corp., Los Angeles, CA) in Krebs–Henseleit buffer containing 0.2% glucose for 10–15 min. Following this period of stabilization, the fluorocarbon emulsion was replaced with oxygenated Krebs–Henseleit buffer containing 0.2% glucose, 5 mM glutamate, 5 mM pyruvate, and 10 mM *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (HEPES). Perfusions with this medium were for periods of 3–5 min and were single pass. To determine the steady-state uptake of fatty acids, the liver was perfused (single pass) with the above Krebs–Henseleit solution to which was added albumin complexed with [^{14}C]palmitate. Various concentrations of the complexes albumin–palmitate and various albumin:palmitate ratios were used. Up to six solutions were perfused sequentially. Uptake of fatty acids was monitored by collecting

effluent fractions every 15 s for 2–3 min (a sufficient time period for steady-state uptake to be established) and assaying for [^{14}C]palmitate.

Viability of the livers throughout the experiments was assessed by monitoring bile flow, which averaged 5 μL/min, by light and electron microscopy, and monitoring effluent aspartate aminotransferase, alanine aminotransferase, and alkaline phosphatase activities. Rat liver microsomal fatty-acyl-CoA ligase was assayed as described previously (Noy & Zakim, 1985a).

Proposed Model

We propose that in the steady state there is an equilibrium distribution of fatty acids between albumin in the vascular space of the liver and the phospholipids of the plasma membranes of all cells in the liver. Moreover, we propose that the rate of uptake of fatty acids by perfused liver, in the steady state, is determined by the partition coefficient for the distribution of fatty acids between these phases. Therefore, we propose that the rate of uptake of fatty acids by liver, and very likely in other tissues, is limited by thermodynamic, not kinetic, factors. In the following discussion, we consider all possible steps in the process of transfer of fatty acids from plasma to their sites of metabolism within liver cells.

Fatty acids are not soluble in water, but they are soluble in the phospholipid regions of biological membranes. Since fatty acids circulate in blood as complexes with albumin, they will be distributed primarily between albumin (alb) in blood and the phospholipid regions of plasma and intracellular membranes. The transfer of a fatty acid (FA) from blood to the interior of a hepatocyte can be described, therefore, by the sequence of reactions 1–4. The terms bilayer$_o$ and bilayer$_i$

$$\text{alb–FA} + n\text{H}_2\text{O} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{alb} + \text{FA–(H}_2\text{O)}_n^{\text{plasma}} \quad (1)$$

$$\text{bilayer} + \text{FA–(H}_2\text{O)}_n \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \text{bilayer}_o\text{–FA} + n\text{H}_2\text{O} \quad (2)$$

$$\text{bilayer}_o\text{–FA} \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} \text{bilayer}_i\text{–FA} \quad (3)$$

$$\text{bilayer}_i\text{–FA} + n\text{H}_2\text{O} \underset{k_2}{\overset{k_{-2}}{\rightleftharpoons}} \text{bilayer} + \text{FA–(H}_2\text{O)}_n^{\text{cytosol}} \quad (4)$$

refer respectively to the outer and inner halves of the phospholipid bilayer of the plasma membrane. Subsequent to entry into the cell, fatty acids will distribute into different intracellular membranes (reaction 5), become bound to proteins (reaction 6), and be metabolized (reaction 7). Reaction 7

$$\text{FA–(H}_2\text{O)}_n^{\text{cytosol}} + \text{intracellular membranes} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \text{membranes–FA} + n\text{H}_2\text{O} \quad (5)$$

$$\text{FA–(H}_2\text{O)}_n^{\text{cytosol}} + \text{protein} \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} \text{protein–FA} + n\text{H}_2\text{O} \quad (6)$$

$$\text{FA} + \text{ATP} + \text{CoA} \xrightarrow{k_7} \text{FA–CoA} + \text{AMP} \quad (7)$$

is the only one considered in the metabolism of fatty acids. All fatty acids are metabolized via eq 7, except for the small amount that undergoes ω-oxidation, and although it is not certain that eq 7 is the rate-setting step in the metabolism of long-chain fatty acids, the flux through reaction 7, in the steady state, must be equal to the total flux of fatty acids through subsequent reactions (oxidation, esterification). Thus, intermediates do not accumulate in these pathways.

Table I: Rate Constants of Reactions Involved in the Palmitate Uptake Process[a]

reaction	rate constant	value of rate constant (s^{-1})
1	k_1	0.036
	k_{-1}	$k_{-1} >> k$
2	k_2	$k_2 >> k_{-2}$
	k_{-2}	7.4
3	k_3, k_{-3}	$\geq$7.4
4	k_{-2}	$\geq$7.4
	k_2	$k_2 >> k_{-2}$
7	V_{max}/K_m	0.71

[a] The values of k_1 and k_{-2} are from Daniels et al. (1985). k_{-1} and k_2 are diffusion-controlled processes (Daniels et al., 1985). k_3 and k_{-3} are expected to be faster than k_{-2} (see text). V_{max}/k_m for palmitoyl-CoA ligase was measured as described under Materials and Methods by using physiological concentrations of ATP and CoA (Siess et al., 1978) and extrapolating to infinite palmitate concentration.

An alternate pathway to reactions 1–4 has been proposed (reaction 8) (Weisiger et al., 1981). The putative membrane

$$\text{alb–FA} + \text{membrane receptor} \rightarrow \text{alb} + \text{FA}_H \quad (8)$$

receptor in eq 8 is considered specific for albumin and is alledged to catalyze dissociation of the complex albumin–FA and thereby to facilitate hepatic uptake into the hepatocyte. FA_H refers to fatty acid at an unspecified location in the hepatocyte.

The first question we considered was the values for the rate constants in reactions 1–7. These are listed in Table I for palmitate as the fatty acid. Rate constants applicable to reactions 1, 2, and 4–7 have been measured directly by using albumin (reaction 1) or synthetic bilayers (reactions 2, 4, 5, and 6). The rates of hydration of palmitate (or other fatty acids) could depend on the exact phospholipid composition of different membranes in which a fatty acid is dissolved (Daniels et al., 1985; DeKruijff & Wirtz, 1977). The rate constants for reactions 2 and 4–6 in cellular membranes could be somewhat different, therefore, from those in Table I. For reasons given below, we do not think that such differences will affect the proposed model.

There are few direct data measurements of rates of transbilayer movement of water-insoluble molecules. There are some data, however, for phospholipids and cholesterol. These molecules cross the bilayer relatively slowly on the time scale of metabolic events (DeKruijff & Wirtz, 1977; Pozansky & Lange, 1976; Donohue-Rolfe & Schaechter, 1980; Seigneuret & Devaux, 1984; Shaw & Thompson, 1982). It is important to note, however, that the rate of transbilayer movement of these molecules is enhanced by adding integral membrane proteins to synthetic bilayers (DeKruijff et al., 1978; Van Zoelan et al., 1978; Gerritsen et al., 1980). With regard to fatty acids, the rate of flip-flop across lipid bilayers is at least as fast as rates of hydration of fatty acids bound to the bilayers (reaction 2). The evidence for this is that the rate of hydration of fatty acids that have been cosonicated into unilamellar vesicles of phosphatidylcholine follows a single time course (Daniels et al., 1985). Since the proteins of a natural membrane can be expected to enhance spontaneous rates of flip-flop (DeKruijff et al., 1978; Van Zoelan et al., 1978; Gerritsen et al., 1980), we think it is reasonable to conclude that the values of k_3 and k_{-3} are at least on the order of those for k_2, and thus that the spontaneous rates of reactions 1, 2, and 4–6 are slower than the spontaneous rate of eq 3. For reaction 7, k_7 is the second-order rate constant for the acyl-CoA ligase assayed at physiologic concentrations of ATP and CoA. The data were extrapolated to infinite concentrations of palmitate.

The expressions for calculating the rates of the reactions important for uptake have the form of eq 9 or 10. Equation

$$v = k[\mathrm{FA}]_n \quad (9)$$

$$v = k[\mathrm{FA}]_{er}[\mathrm{E}] \quad (10)$$

9 applies to reactions 1–6; $[\mathrm{FA}]_n$ is the concentration (mole fraction) of fatty acid in membrane or albumin phases. Equation 10 applies to reaction 7. $[\mathrm{FA}]_{er}$ is the concentration of fatty acid in the hepatic endoplasmic reticulum, and [E] is the concentration of the enzyme in the same membrane. Since we have shown previously that acyl-CoA ligase interacts with fatty acids that are dissolved in the membrane not in water (Noy & Zakim, 1985b), the rate of eq 7 is related to the concentration of fatty acid in the endoplasmic reticulum or in mitochondria.

The important conclusion to be drawn from the data in Table I is that events of distribution and metabolism of fatty acids within the cell cannot be separated from those outside the cell. This is so because k_3 and k_{-3} are large as compared with rate constants for steps in the uptake process. The rate constants available for estimating rates of reactions 1–7 in the liver suggest, therefore, that fatty acids bound to albumin in blood will be equilibrated with fatty acids bound to the plasma membranes. The concentration of fatty acids in the plasma membrane will determine the rate of hydration of fatty acid in cell water just inside the cell and hence the concentration of fatty acids in this limited region of the cytosol. This concentration will determine, in turn, the rate of diffusion of fatty acids from the plasma membrane to the water surrounding the endoplasmic reticulum or mitochondria (the locations of the ligase), the concentration of fatty acids in the membranes of these organelles, and by eq 10 the rate of reaction 7. Since uptake of fatty acids by liver in the steady state must equal flux in each individual step of the process, e.g., transbilayer movement, diffusion from plasma membrane to endoplasmic reticulum, metabolism catalyzed by acyl-CoA ligase, etc., the model predicts that uptake of fatty acids in the steady state will be determined by the concentration of fatty acid in endoplasmic reticulum and mitochondria, which will depend in turn on the equilibrium distribution of FA between albumin and plasma membranes. Rates of uptake should be sensitive, therefore, to perturbations of this equilibrium.

Results

Evidence That There Is an Equilibrium Distribution of Palmitate between Albumin and Plasma Membranes in the Steady State. We want to know first of all whether the rate of eq 1 is sufficiently rapid that the partitioning of fatty acids between albumin in plasma and the plasma membranes of cells will be at thermodynamic equilibrium, under physiologic conditions of perfusion. We need to determine, therefore, the amount of fatty acid that can dissociate from albumin per minute per gram of liver and the amount of fatty acid that must be transferred from albumin to plasma membranes to achieve an equilibrium distribution of fatty acids between these two compartments.

The amount of palmitate that can dissociate from albumin per minute per gram of liver can be calculated from eq 11.

$$\text{amount of palmitate dissociated} = k_1 \bar{t} F[\text{palmitate}] \quad (11)$$

k_1 is the rate constant of reaction 1, $\bar{t}$ is the mean transit time of albumin in liver, F is the flow rate of the perfusate (in milliliters per minute per gram of liver), and [palmitate] is the concentration of palmitate in the perfusate. The rate constant, k_1, is 0.036 s^{-1} at 37 °C, under the condition that palmitate is the fatty acid and the initial value of the ratio moles of palmitate to moles of albumin is 2 (Daniels et al., 1985). This last qualification is important because albumin has different classes of binding sites for fatty acids, based on the affinity of binding (Ashbrook et al., 1975). The mean transit time ($\bar{t}$) of albumin in liver is about 14 s at a physiologic rate of blood flow, which is 1.5 mL min^{-1} (g of liver)$^{-1}$ (Fischer, 1963). When [palmitate] is 0.5 mM and the palmitate:albumin ratio is 2, the total amount of palmitate that can dissociate is 0.4 μmol/min^{-1} (g of liver)$^{-1}$. That is, under the conditions given, more than 50% of the albumin-bound palmitate can dissociate from albumin during a single pass through the liver. Whether or not this amount of palmitate actually dissociates will depend on factors independent of k_1, $\bar{t}$, and F.

The equilibrium distribution (molal basis) of palmitate between albumin and unilamellar bilayers of phosphatidylcholine from egg, when the palmitate:albumin ratio at equilibrium is 2, was measured and found to be 0.00260, in favor of the albumin complex (Daniels et al., 1985; Figure 2). We assume that the equilibrium constant will be 1 or close to 1 for the distribution of palmitate between unilamellar bilayers of phosphatidylcholine and plasma membranes. The volume accessible to albumin per gram of liver (in the steady state) was calculated from the flow rate of blood through the liver and the mean transit time of albumin in liver. This volume was estimated to be 0.35 mL/g of liver (see below). Hence, when the concentration of albumin in perfusate is 0.25 mM, there are 8.75 μmol of albumin/g of liver. The amount of palmitate that would have to be transferred from albumin to the membranes of liver cells, to achieve an equilibrium distribution of palmitate in these phases, can be calculated from eq 12. K_{eq} is the equilibrium constant for the distribution of

$$K_{eq} = \frac{X/\text{mol of lipid}}{([\text{palmitate}]_{total} - X)/\text{mol of albumin}} \quad (12)$$

palmitate between lipid bilayers and albumin; $[\text{palmitate}]_{total}$ is the amount of palmitate per gram of liver when the palmitate:albumin ratio equals 2; X is the amount of palmitate associated with membranes per gram of liver. The area of all plasma membranes of liver cells is estimated to be about 3300 cm^2/g of liver (Blouin et al., 1977). Since the sinusoidal membranes constitute about 70% of plasma membranes in liver (Blouin et al., 1977), we calculate that there are 660 nmol of sinusoidal phospholipid/g of liver. This calculation is based on the area per phospholipid molecule in a bilayer structure in reference (Worcester, 1976). This calculated amount of phospholipid in sinusoidal membranes is an overestimate because it does not consider the surface area due to proteins. The overestimate has no importance consequence for the development of the model, however.

It is found from eq 12, under the conditions given, that 3.6 nmol of palmitate must be transferred from albumin to the plasma membranes, or about 2% of palmitate bound initially to albumin, in order to achieve an equilibrium distribution of palmitate between albumin and plasma membranes. Since the rate constant for dissociation of palmitate from albumin and the mean transit time for albumin in liver allow for the dissociation of more than 50% of the palmitate bound to albumin during a single passage through the liver, an equilibrium distribution of palmitate between albumin and plasma membranes will be achieved within a small fraction of the time it takes albumin to circulate through the liver.

Relationship between Observed Rates for Uptake of Palmitate by Perfused Liver and the Rate of Reaction 1. The next set of experiments was carried out to determine whether k_1 (reaction 1) was sufficiently rapid to account for the ob-

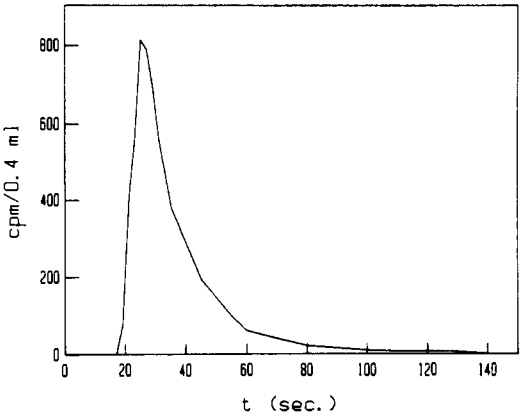

FIGURE 1: Outflow dilution curve for albumin passing through a representative, perfused liver. The liver was prepared and perfused by the technique outlined in the text. 0.7 mL of a 0.25 mM ^{14}C-methylated albumin solution was rapidly injected into the liver through the portal vein. The liver then was perfused with 0.25 mM albumin solution, and fractions were collected every 3 s up to 120 s.

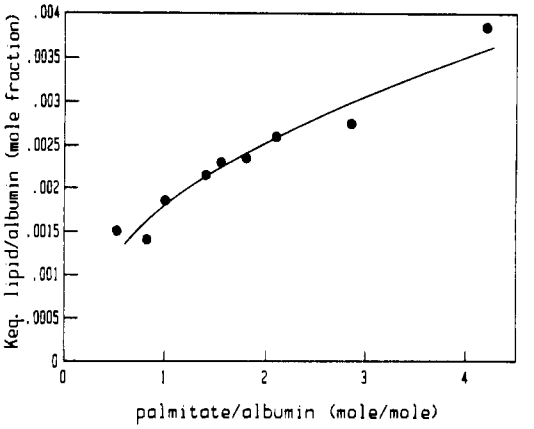

FIGURE 2: Equilibrium constant for distribution of palmitate between lipid bilayers and albumin. Solutions of 0.2 mM albumin containing bound [^{14}C]palmitate at various palmitate:albumin ratios were mixed with unilamellar vesicles of egg phosphatidylcholine. The distribution of palmitate between albumin and the vesicles was determined as described under Materials and Methods. The equilibrium constant is the ratio moles of palmitate (in vesicles)/moles of lipid to moles of palmitate (in albumin)/moles of albumin.

served rates of uptake of palmitate by perfused liver. It remained a possibility that the attainment of equilibrium in the partitioning of palmitate between albumin and plasma membranes was not relevant to the uptake of palmitate into the liver and that some mechanism other than reactions 1–4 might be required to explain the uptake process, as for example reaction 8. In order to determine whether k_1 was rapid enough to account for the uptake of palmitate in a perfused liver, we measured the mean transit time for albumin in a perfused liver, in addition to uptake rates.

The outflow dilution curve for albumin passing through a representative perfused liver is shown in Figure 1. As expected, there was an initial delay in the outflow of albumin followed by a rapid rise to a peak and then a slow decay. To validate the estimate of mean transit time from this curve, it has to be shown that all of the indicator put into the liver is recovered at the outflow (Goresky & Rose, 1977). This can be shown by the calculation that 98.5% of the counts of 0.25 mM ^{14}C-methylated albumin solution injected into the liver were recovered by 120 s. The observed mean transit time ($\bar{t}_{obsd}$) can be calculated from eq 13, in which t is the time (in seconds) after injection up to 120 s, $C(t)$ is the concentration of ^{14}C-methylated albumin in each fraction, and Δt = 3 s.

$$\bar{t}_{obsd} = \frac{\int_0^{120} tC(t)\,dt}{\int_0^{120} C(t)\,dt} \approx \frac{\sum_1^{t/\Delta t} tC(t)\Delta t}{\sum_1^{t/\Delta t} C(t)\Delta t} \qquad (13)$$

The value of $\bar{t}_{obsd}$, for the experiment depicted in Figure 1, was 31.2 s. At the flow rate used in Figure 1, the transit time through the input and collecting systems ($\bar{t}$ system) was 23 s; so the mean transit time through the liver was 8.2 s (eq 14).

$$\bar{t}_{liver} = \bar{t}_{obsd} - \bar{t}\text{ system} = 8.2\text{ s} \qquad (14)$$

The average mean transit time was 8.5 ± 0.37 s per liver (N = 4). Normalizing to a physiological rate of blood flow, which is about 1.5 mL min^{-1} (g of liver)$^{-1}$ (Fischer, 1963), gives a mean transit time of albumin through the liver of about 14 s. This value agrees well with what has been found in an intact dog (Goresky & Rose, 1977).

The fraction of fatty acid extracted during a single pass through the liver is given by

$$\text{extraction fraction} = k_1\bar{t}_{liver} \qquad (15)$$

The maximal rate of uptake can be calculated by using

$$\text{uptake rate} = k_1\bar{t}_{liver}F[\text{FA}] \qquad (16)$$

F in eq 16 is the flow rate in milliliters per minute per gram of liver, and [FA] is the concentration of fatty acid in the perfusate. The rate constant for dissociation of palmitate from albumin (k_1) is 0.036 s^{-1} when the palmitate:albumin ratio is 2 (Daniels et al., 1985). The maximal uptake rate when [FA] is 0.5 mM is thus calculated to be 390 nmol min^{-1} (g of liver)$^{-1}$. The measured steady-state uptake rate of palmitate under these conditions was 120 nmol min^{-1} (g of liver)$^{-1}$ (Figure 3). It is apparent then that the rate of dissociation from albumin cannot limit the uptake of palmitate by the liver. In this respect, it is important to appreciate that the physiological rate of hepatic blood flow is slower than flow rates used in perfusion experiments. The transit time of albumin through the liver under physiological conditions, therefore, is much longer than that observed in Figure 1.

Rate of Uptake of Palmitate by Liver as a Function of the Equilibrium Distribution of Palmitate between Albumin and Plasma Membranes. The proposed model predicts that the uptake of fatty acids by the liver will vary as the equilibrium distribution of fatty acids between albumin and plasma membrane is varied. This equilibrium can be altered because albumin contains different classes of binding sites for fatty acids (Ashbrook et al., 1975). This is illustrated by the equilibrium constants for the distribution of palmitate between unilamellar vesicles of phosphatidylcholine and albumin at various palmitate:albumin ratios as shown in Figure 2. Increasing ratios of palmitate to albumin (at constant albumin concentration) result in an increased partitioning of palmitate into the lipid bilayer phase of the system. The curve obtained bends continuously. At low palmitate:albumin ratios, a condition under which palmitate is bound predominantly to the high-affinity binding sites on albumin (Ashbrook et al., 1975), the change in the partition coefficient as a function of increasing palmitate:albumin ratio is steeper vs. what is observed at high ratios.

The steady-state rates of uptake of palmitate by perfused liver at various palmitate:albumin ratios but a constant concentration of albumin (0.25 mM) were measured. The data, when plotted in double-reciprocal form (Figure 3), reveal that there is no simple relationship between rates of uptake of palmitate and the palmitate:albumin ratio. Instead, uptake rates are continuously variable as a function of this ratio. Especially noteworthy is the steep dependence of the uptake

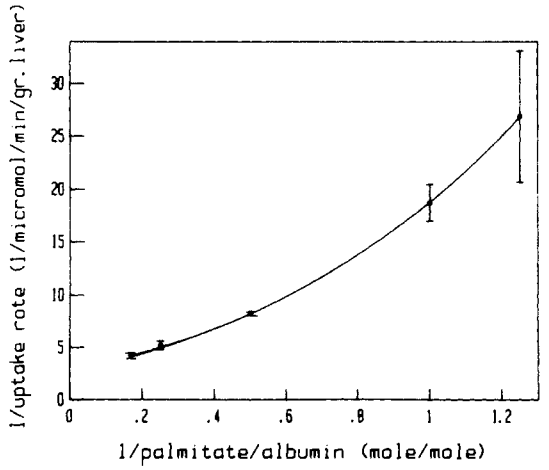

FIGURE 3: Uptake of palmitate by perfused rat liver as a function of the palmitate:albumin ratio. The concentration of albumin in the perfusate was 0.25 mM. Liver perfusions were performed as described under Materials and Methods. Each point is the average of two to three experiments. The data are plotted in double-reciprocal form. The error bars indicate the standard error for each point.

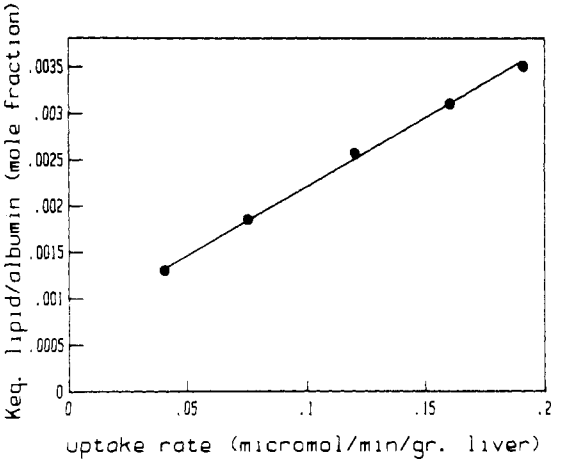

FIGURE 4: Uptake of palmitate by perfused liver as a function of K_{eq} for the distribution of palmitate between lipid bilayers and albumin. The data in Figures 1 and 2, in the range of palmitate:albumin ratios of 0.5–4, were used.

rate on the palmitate:albumin ratio at low values for this ratio and the apparent activation of uptake for relatively large values of this ratio. Measured rates for the uptake of palmitate as a function of different palmitate:albumin ratios are plotted in Figure 4 as a function of K_{eq} for the distribution of palmitate between unilamellar lipid bilayers and albumin. The data in this figure show a linear relationship between the equilibrium distribution of palmitate in the system albumin plus lipid bilayers and the observed rate of uptake of palmitate by perfused rat liver at various palmitate:albumin ratios, under the condition that the concentration of albumin is constant. The line in Figure 4 cannot be extended reliably to lower values of the palmitate:albumin ratio (lower values of K_{eq}) because of the difficulty in measuring accurately low rates of uptake. Nevertheless, the data appear to provide a convincing validation of the predictive value of the model for rates of hepatic uptake of palmitate.

Uptake of Palmitate at a Constant Palmitate:Albumin Ratio but Variable Concentrations of the Complex Albumin–Palmitate. Extrapolation of the data in Figure 3 to an infinite palmitate:albumin ratio suggests that the rate of uptake of palmitate by perfused liver will be finite even when the concentration of fatty acids in perfusate is infinite. Data such as these have been interpreted as evidence for the involvement

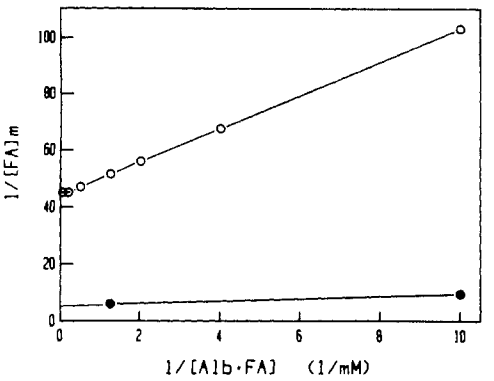

FIGURE 5: Calculated concentrations of palmitate in lipid bilayers at various alb–FA concentrations and fixed FA:albumin ratios. The concentrations in the membranes ($[FA]_m$) were calculated from the equilibrium distribution constants shown in Figure 2 and by using the equation $K_{eq} = \{[FA]_m/[lipid]\}/\{([FA]_{total} - [FA]_m)/[albumin]\}$. The concentration of lipid was taken to be 33 μmol/g of liver, and the concentrations of albumin and palmitate were varied. Two cases are shown: (○) initial FA:albumin ratio = 0.5; (●) initial FA:albumin ratio = 4.0.

of specific mechanisms in the uptake processes for nonpolar compounds. One has to consider, however, the exact dependence of the concentration of fatty acid in membranes on the total amount of the complex albumin–FA at a given fixed ratio of fatty acid to albumin and for different fixed ratios. The importance of this point is illustrated in Figure 5. The concentrations of palmitate in membranes are plotted in this figure as a function of different amounts of complex albumin–fatty acid. The plot is in double-reciprocal form to emphasize that the concentration of fatty acid in membrane does not become infinite at infinite concentrations of the complex (albumin–fatty acid). Moreover, the concentration of fatty acid in membrane when the palmitate:albumin ratio is 0.5 never will be as large as it is when the palmitate:albumin ratio is greater than 0.5. These results lead to important predictions about the kinetic pattern for rates of uptake of fatty acid by liver as a function of the concentration of the complex (albumin–fatty acid) at different fixed palmitate:albumin ratios. The kinetics will appear to be Michaelis–Menten for any fixed ratio of palmitate to albumin, but the maximal rate of uptake should depend on the fixed ratio of palmitate to albumin. In addition, the uptake rate will reach a limiting value at finite values of the concentration of the albumin–fatty acid complex. The first two predictions are validated by the data in Figure 6. Thus, for a given palmitate:albumin ratio, the dependence of the uptake rate on the concentration of the complex albumin–fatty acid conforms to Michaelis–Menten kinetics. Moreover, the maximal attainable rate of uptake at infinite concentrations of complex (albumin–fatty acid) increases as the palmitate:albumin ratio increases. The data for the highest fatty acid:albumin ratio also suggest that the measured uptake rate reaches a limiting value at finite concentrations of the complex.

Does the Model Provide a Flux of Palmitate through Hepatic Cytosol That Is Sufficiently Large To Sustain Observed Rates of Uptake? Uptake of fatty acids by the liver will depend on diffusion from the inner half of the plasma membrane to other cell organelles, in which they will be metabolized. Diffusion could be due simply to movement of fatty acids solvated by water. Alternatively, it has been proposed that movement of fatty acids through cytosol is facilitated by binding to a 12 000-dalton cytosolic protein, which has been designated fatty acid binding protein (Ockner & Manning, 1979; Mishkin & Turcotte, 1974; Burnett et al., 1977). Irrespective of the exact mode of diffusion, one has

Table II: Calculated and Observed Steady-State Fluxes of Palmitate through the Cytosol at Various Palmitate:Albumin Ratios[a]

palmitate:albumin (mol/mol)	$[FA]_{pm}$ [(mol of FA/mol of lipid) × 100]	$[FA]_{er}$ [(mol of FA/mol of lipid) × 100]	$[FA]_{H_2O,pm}$ (M)	$[FA]_{H_2O,er}$ (M)	J [μmol min^{-1} (g of liver)$^{-1}$]	obsd uptake [μmol min^{-1} (g of liver)$^{-1}$]
0.5	0.0625	0.0026	7×10^{-9}	0.03×10^{-9}	0.018	0.04
2	0.52	0.009	58×10^{-9}	0.9×10^{-9}	0.143	0.12
4	1.4	0.018	155×10^{-9}	2.0×10^{-9}	0.39	0.33

[a] The concentrations of palmitate in plasma membranes ($[FA]_{pm}$) were calculated from the distribution constant of palmitate between albumin and lipid bilayers (Figure 2). The concentrations of palmitate in the endoplasmic reticulum membrane ($[FA]_{er}$) were calculated from the double-reciprocal relation of fatty-acyl-CoA ligase activity vs. fatty acid concentration when the velocity of the enzymatic reaction equals the uptake rate (steady-state conditions). The enzyme was assayed as described under Materials and Methods at physiological concentrations of ATP and CoA (Siess et al., 1978). The concentrations of palmitate in the cytosol, at the plasma membranes ($[FA]_{H_2O,pm}$) and at the endoplasmic reticulum ($[FA]_{H_2O,er}$), were calculated by using 5×10^6 for the equilibrium distribution of palmitate between lipid bilayers and water. J was calculated from eq 17. The observed uptake rates were measured as described under Materials and Methods.

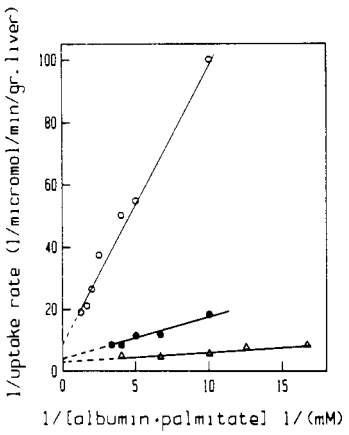

FIGURE 6: Uptake of palmitate by perfused rat livers when the palmitate:albumin ratio is constant. The ratio of palmitate to albumin in the perfusate was 0.5 (○), 2.0 (●), or 4 (△). The concentration of the palmitate–albumin complex was varied. The data were plotted in double-reciprocal form.

to consider whether the concentrations of fatty acids in the plasma membrane, which according to our model are determined only by the values of k_1 and k_2 (reaction 2), are high enough to sustain rates of diffusion equal to observed rates of uptake of fatty acids, for it is obvious that these rates must be equal in the steady state. Diffusion fluxes (J) can be calculated from

$$J = \bar{D}(\Delta C/\Delta X) \qquad (17)$$

D, the diffusion coefficient of palmitate in water, is taken to be 6×10^{-6} cm^2/s (Sallee & Dietchy, 1973). ΔX, the diffusion path, is taken to be 0.5×10^{-3} cm, the average radius of the hepatocyte (Tipping & Ketterer, 1981). The problem in solving J is to calculate ΔC, which is the concentration gradient of fatty acid between cytosol adjacent to the inner half of the plasma membrane vs. cytosol adjacent to endoplasmic reticulum and mitochondria. The maximum gradient possible will occur when fatty acid in the plasma membrane is at equilibrium with albumin in sinusoids and there is no fatty acid anywhere else in the hepatocyte. When the ratio of palmitate to albumin is 4, the equilibrium concentration of fatty acids in phospholipids in the plasma membrane is 1.4 mol %. On the basis of the partitioning of palmitate between water and phospholipid membranes, the aqueous phase concentration of palmitate in water adjacent to the inner side of the plasma membrane will be 155×10^{-9} M. Assuming no palmitate in the endoplasmic reticulum and a distance of 0.5×10^{-3} cm between plasma membrane and endoplasmic reticulum (or mitochondria), the flux between these compartments will be 400 nmol min^{-1} (g of liver)$^{-1}$. This value for the flux of palmitate compares with an uptake rate (Figure 3) of 330 nmol of palmitate min^{-1} (g of liver)$^{-1}$. Hence, the maximal flux of palmitate through the cytosol is somewhat larger than uptake. The flux of 400 nmol has to be corrected, however, for the concentration of palmitate in cytosol adjacent to sites of metabolism of palmitate. These concentrations can be calculated from eq 7 and the partition coefficient for palmitate between membrane and water because the flux of fatty acid through the ligase-catalyzed step must equal the flux through all other steps in the uptake process. Measurement of the kinetic constants for the ligase using concentrations of CoA and ATP that exist in cytosol in vivo makes it possible to calculate the concentration of palmitate, in membranes containing acyl-CoA ligase, needed to sustain observed rates of uptake. This concentration is 0.018 mol % for an uptake rate of 330 nmol min^{-1} g^{-1}. The concentration of palmitate in water adjacent to a membrane containing this much palmitate will be 2×10^{-9} M. Flux between the plasma membrane and endoplasmic reticulum and mitochondria, therefore, will be 390 nmol min^{-1} (g of liver)$^{-1}$. In other words, the calculated flux of palmitate through cytosol, assuming no special mechanisms for concentrating palmitate in plasma membranes or for facilitating the transport of palmitate in cytosol, is essentially identical with observed rates of uptake for palmitate. Experimentally determined and calculated values for various rate constants, concentrations of palmitate in different regions of the hepatocyte, and flux through the cytosol are shown in Table II for palmitate at ratios of moles of palmitate to moles of albumin different from 4. The importance of these data is the close correspondence between calculated values for J as compared with observed rates of uptake. We want to emphasize that the calculations of J are based only on measurements of kinetic constants of the acyl-CoA ligase and the equilibrium distribution of fatty acids between membranes and albumin and between membranes and water. Obviously, the exact relationship between the calculated values for J and real events in vivo is uncertain because of the assumptions on which the calculated values are based, for example, that all the acyl-CoA ligase is equidistant from the plasma membrane. Obviously, the acyl-CoA ligase is present also in mitochondria. The most important aspect of the calculated data in Table II, however, is that the values of J are of the proper order of magnitude to account for observed rates of uptake of palmitate without postulating specific mechanisms for facilitating this process.

We considered for the purpose of the above analysis whether fatty acids in hepatic cytosol would be bound to the putative fatty acid transport protein and whether such binding would facilitate diffusion. On the basis of data in the literature for the affinity of the protein for fatty acids, its abundance in liver (Ockner & Manning, 1979; Mishkin & Turcotte, 1974; Burnett et al., 1977), and the calculated concentrations of fatty acid in cytosol at the inner half of the bilayer, we calculate that less than 0.1% of total fatty acids in cytosol will be bound

Table III: Uptake of Fatty Acids by Perfused Rat Livers[a]

fatty acid	obsd uptake rate [μmol min^{-1} (g of liver)$^{-1}$]	calcd max uptake [μmol min^{-1} (g of liver)$^{-1}$]	k_1 (s^{-1})
14:0	0.062 ± 0.01[b]	1.3	0.119
16:0	0.067 ± 0.0035[b]	0.39	0.036
18:0	0.063 ± 0.007[c]	0.11	0.0102

[a] Livers were perfused with solutions containing 0.25 mM bovine serum albumin and 0.5 mM ^{14}C-labeled fatty acid. Steady-state rates of uptake were calculated from analyses of effluent samples. Values are given as mean ± SEM. Maximal rates of uptake were calculated from the expression $k_1\bar{t}_{liver}F[FA]$ where k_1 is the first-order rate constant for dissociation of the complex albumin–fatty acid, $\bar{t}_{liver}$ is the mean transit time of albumin through the liver, F is the perfusion flow rate in milliliters per minute per gram of liver, and [FA] is the fatty acid concentration in the perfusate (millimolar). The values for k_1 are taken from Daniels et al. (1985). [b] $N = 3$. [c] $N = 6$.

to the fatty acid binding protein. Since the rate of diffusion of this binding protein cannot exceed that for the free fatty acids, it is clear that flux via the fatty acid binding protein will be insignificant. Therefore, this mechanism for cytosolic transport was not considered important.

Do Rates of Uptake of Fatty Acids Other Than Palmitate Agree with the Proposed Model? Since the affinity of binding of fatty acids to albumin, the first-order rate constant for dissociation of fatty acids from albumin, and the rates of metabolism depend on the structure of a fatty acid (Noy & Zakim, 1985a,b; Daniels et al., 1985; Ashbrook et al., 1975), we measured the steady-state uptake rate for two fatty acids, in addition to palmitate, that differ widely in rates of dissociation from albumin. The additional fatty acids examined were myristate and stearate. The experiments in Table III were done by using a different population of rats from experiments described above. The latter population was about 4 months older than those used in previous experiments. Possibly, this age difference accounted for the somewhat different values of uptake rates obtained for palmitate in these vs. previous experiments. Nevertheless, observed uptake rates for myristate and stearate agree with conclusions reached from the uptake of palmitate. The rate constants for dissociation from albumin (k_1) and the observed steady state of uptake of the three fatty acids by liver are shown in Table III. Calculated maximal uptake rates also are shown. It is apparent from the data in Table III that the rate of dissociation of fatty acids from albumin cannot limit the uptake of fatty acids by the liver. It is noteworthy that even the very slow rate of dissociation of stearate from albumin does not limit the observed rates of hepatic uptake of this fatty acid.

As discussed above, the concentrations of fatty acids in the plasma membranes are determined by their equilibrium distribution between albumin and these membranes. The flux through the cytosol then is determined by the concentration gradient of fatty acids between the plasma membranes and the endoplasmic reticulum (or mitochondria) membranes. This gradient, in turn, is determined by the catalytic properties of fatty-acyl-CoA ligase with the various fatty acids. The uptake rate, therefore, should be (linearly) related to the product of the lipid–albumin distribution constant and the rate constant for reaction 7. The relevant rate constants for the three fatty acids used (under the condition that the FA:albumin ratio is 2) are shown in Table IV. The calculated values of K_{eq} in Table IV predict that the uptake rates of the three fatty acids will be nearly the same. Measurements of uptake rates of myristate, palmitate, and stearate agree with this prediction (Table III).

Discussion

It is assumed, in general, that the uptake into cells of hydrophobic compounds is mediated by specialized membrane components such as transport proteins and/or specific receptor proteins. The evidence for specialized transport mechanisms across membranes for uptake of water-insoluble compounds is indirect, however. The general state of this area is exemplified by the literature on fatty acid metabolism. For example, it is believed that specific receptors and membrane transport proteins mediate cellular uptake of fatty acids because uptake is saturable and can be inhibited by fatty acids, phospholipases, phloretin, analogues of fatty acids, or agents that react with protein (Mahadevan & Sauer, 1971, 1974; Samuel et al., 1976; Weisiger et al., 1981; Abumrad et al., 1981, 1984). Consideration of how fatty acids distribute between albumin and bilayers shows that saturation of uptake of a water-insoluble compound cannot be invoked as evidence for specific transport systems. In addition, none of the experiments in which uptake was inhibited excluded the possibilities that the nonspecific inhibitors altered the metabolism or the partitioning of fatty acids into membranes of the cells (Mahadevan & Sauer, 1971, 1974; Samuel et al., 1976; Abumrad et al., 1981, 1984). We believe, therefore, that current ideas about the cellular uptake of water-insoluble substances are not based on clear-cut evidence and were led to a consideration of the basic events in the interactions of water-insoluble substances with membranes and albumin.

The initial step in the uptake of fatty acids by mammalian cells will be the transfer of fatty acids from complexes of albumin–FA in serum to the outer membrane of cells. The data presented in the present work show that this step is rapid enough in liver to be at equilibrium. The kinetics of fatty acid uptake by liver can be understood completely on the basis of this idea. No specialized biochemical mechanisms need to be postulated in order to understand the process. Moreover, the data presented above indicate that control of the process of fatty acid uptake by liver can be achieved simply on the basis of the chemical structure of serum albumin and biological membranes and their relative affinities for fatty acids. What is interesting too about the data is that they provide a basis for understanding how uptake of fatty acids can be regulated in the absence of specific mechanisms for the uptake process. First, calculations based on the model show that fatty acids do not become distributed widely inside the cell. This is so because aqueous phase concentrations just inside the cell are

Table IV: Predicted and Observed (Relative) Uptake Rates of Myristate, Palmitate, and Stearate by Rat Liver[a]

fatty acid	K_{eq}, lipid:albumin	V_{max}/K_m (min^{-1})	data normalized to palmitate			obsd uptake rate [μmol min^{-1} (g of liver)$^{-1}$]
			K_{eq}	V_{max}/K_m	$K_{eq}(V_{max}/K_m)$	
palmitate (16:0)	0.0026	42.7	1	1	1	0.067
myristate (14:0)	0.002	47.6	0.76	1.1	0.86	0.062
stearate (18:0)	0.012	6.5	4.62	0.152	0.7	0.063

[a] The distribution constants of fatty acids between lipid bilayers and albumin were measured, and the rate constants for fatty-acyl-CoA ligase (V_{max}/K_m) were determined as described under Materials and Methods. Uptake rates are predicted to be related to the product $K_{eq}(V_{max}/K_m)$ (see text).

low, which limits diffusion. In addition, acyl-CoA ligase, because it functions within membranes that concentrate the fatty acid substrate, has relatively high catalytic activity at extraordinarily low concentrations of fatty acids in cytosol. Thus, the simple physical–chemical partitioning of fatty acid between plasma membranes and plasma albumin seems to provide the system with sufficient regulatory properties to prevent uptakes of fatty acids that exceed metabolic capacity. Second, the process of uptake also is subject to dynamic regulation by factors that alter the partitioning of fatty acids between albumin and bilayers and by changes in the amount or catalytic efficiency of acyl-CoA ligase.

The simplest way to alter hepatic uptake of fatty acids is to change the ratio of moles of fatty acid bound per mole of albumin. As this ratio increases, fatty acids are bound at sites with decreasing affinity, which shifts the equilibrium between membranes and albumin in favor of the membrane (Figure 2). The data in Figures 3–6 indicate how this affects rates of uptake. Animals appear to regulate hepatic uptake of fatty acids by increasing or decreasing the occupation of low-affinity binding sites on albumin. In the normal fed state, for example, rates of metabolism of fatty acids are relatively low, and the ratio in blood of moles of fatty acids per mole of albumin is about 0.5. When animals are fasted, this ratio increases, and hepatic uptake of fatty acids increases as do rates of fatty acid oxidation in other tissues. Another example of the compatibility of the model with physiologic data is that observed rates of oxidation of fatty acids in liver were found to be greater in patients with cirrhosis vs. those with normal liver functions (Owen et al., 1983). Both groups were studied after an overnight fast. The patients with cirrhosis had an average of 1.96 mol of fatty acids per mole of albumin in blood. This ratio was 0.89 in patients without liver disease. The partition coefficient for the distribution of fatty acids between albumin and the membranes of cells, therefore, would have been larger in the group with cirrhosis. This fact alone would account for the greater rates of fatty acid oxidation in the patients with cirrhosis vs. the normal group. Changes in the activity of ligase also might alter rates of uptake of fatty acids, but the extent of such changes will be limited by diffusion within the cell.

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Registry No. Palmitoyl-CoA, 1763-10-6; stearic acid, 57-11-4; myristic acid, 544-63-8; palmitic acid, 57-10-3.

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