Hoylaerts, M., Rijken, D. C., Lijnen, H. R., & Collen, D. (1982) *J. Biol. Chem. 257*, 2912-2919.
Juhan-Vague, I., Calas, M. F., Roux, F., Juhan, C., Durand-Dessemon, F., deLaforte, C., & Serradimigni, A. (1981) *Thromb. Haemostasis 45*, 154-157.
Laemmli, U. K. (1972) *Nature (London) 227*, 680-685.
Lijnen, H. R., Hoylaerts, M., & Collen, D. (1980) *J. Biol. Chem. 255*, 10214-10222.
Lorand, L., & Middlebrook, W. R. (1952) *Biochem. J. 52*, 196-199.
Lucas, M. A., Fretto, L. J., & McKee, P. A. (1983a) *J. Biol. Chem. 258*, 4249-4256.
Lucas, M. A., Straight, D. L., Fretto, L. J., & McKee, P. A. (1983b) *J. Biol. Chem. 258*, 12171-12177.
Markus, G., Evers, J. L., & Hobika, G. H. (1978) *J. Biol. Chem. 253*, 733-739.
Melhado, L. L., Peltz, S. W., Leytus, S. P., & Mangel, W. F. (1982) *J. Am. Chem. Soc. 104*, 7299-7306.
Mihalyi, E. (1983) *Ann. N.Y. Acad. Sci. 408*, 60-70.
Moroi, M., & Aoki, N. (1976) *J. Biol. Chem. 251*, 5956-5965.
Moroi, M., & Aoki, N. (1977) *Thromb. Res. 10*, 851-856.
Panyim, S., & Chalkley, R. (1969) *Arch. Biochem. Biophys. 130*, 337-346.
Peltz, S. W., Hardt, T. A., & Mangel, W. F. (1982) *Biochemistry 21*, 2798-2804.
Rabiner, S. F., Goldfine, I. D., Hart, A., Summaria, L., & Robbins, K. C. (1969) *J. Lab. Clin. Med. 74*, 265-273.
Rákóczi, I., Wiman, B., & Collen, D. (1978) *Biochim. Biophys. Acta 540*, 295-300.
Robbins, K. C., Summaria, L., Hsieh, B., & Shah, R. (1967) *J. Biol. Chem. 242*, 2333-2342.
Schultze, H. E., & Heremans, J. F. (1966) *Molecular Biology of Human Proteins with Special Reference to Plasma Proteins*, Vol. 1, p 177, Elsevier, Amsterdam.
Shah, G. A., & Dhall, D. P. (1983) *Thromb. Res. 32*, 67-72.
Sherry, S. (1968) *Annu. Rev. Med. 19*, 247-268.
Suenson, E., & Thorsen, S. (1981) *Biochem. J. 197*, 619-628.
Takada, A., & Takada, Y. (1981) *Thromb. Res. 22*, 497-501.
Takada, A., Urano, T., & Takada, Y. (1979) *Thromb. Haemostasis 42*, 901-908.
Takada, A., Takada, Y., & Sugawara, Y. (1984) *Thromb. Res. 33*, 561-569.
Thorell, J. I., & Johansson, B. G. (1971) *Biochim. Biophys. Acta 251*, 363-369.
Thorsen, S. (1975) *Biochim. Biophys. Acta 393*, 55-65.
Wallén, P. (1980) in *Fibrinolysis* (Kline, D. L., & Reddy, K. N. N., Eds.) pp 1-24, CRC Press, Boca Raton, FL.
Wallén, P., & Wiman, B. (1972) *Biochim. Biophys. Acta 257*, 122-142.
Walther, P. J., Hill, R. L., & McKee, P. A. (1975) *J. Biol. Chem. 250*, 5926-5933.
Weinstein, M. J., & Doolittle, R. F. (1972) *Biochim. Biophys. Acta 258*, 577-590.
Whitaker, A. N., Rowe, E. A., Masci, P. P., Joe, F., & Gaffney, P. J. (1980) *Thromb. Res. 19*, 381-391.
Wiman, B., & Wallén, P. (1977) *Thromb. Res. 1*, 213-222.
Wiman, B., & Collen, D. (1978) *Nature (London) 272*, 549-550.

Rates of Hydration of Fatty Acids Bound to Unilamellar Vesicles of Phosphatidylcholine or to Albumin†

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ABSTRACT: The rates of hydration of naturally occurring fatty acids bound to unilamellar vesicles of dimyristoylphosphatidylcholine were measured by following the rate of quenching of the inherent fluorescence of albumin. Rates of hydration of fatty acids bound to albumin could be estimated from the same data. The data show that these rates depend on the chain length and unsaturation of the fatty acid. Increasing chain length diminishes the rate of hydration whereas increasing unsaturation increases this rate. Rates of hydration of fatty acids bound to lipid vesicles appear to be rapid enough to account for intracellular movement between compartments in the absence of carrier proteins. It is uncertain whether this is true for hydration of fatty acids bound to albumin. Rates for this process are about 100-300 times slower vs. rates of hydration of fatty acids bound to lipid vesicles.

The pathways by which water-insoluble compounds move between different compartments within cells are uncertain. It has been proposed for several such compounds that they are transported between intracellular compartments as complexes with carrier proteins, the latter having variable specificities for binding of water-insoluble ligands. Some of the putative transport proteins have been shown directly in vitro to catalyze transport of phospholipids, cholesterol, and tocopherol between membranous compartments separated by an aqueous phase (Bloj & Zilversmit, 1977; Catignani & Bieri, 1977; Hellings et al., 1976; Kamp et al., 1973; Noland et al., 1980; Yamada & Sasaki, 1982). It is assumed but unproved, on the other hand, that intracellular transport proteins are essential for movement through water for many other organic, hydrophobic compounds (Ketterer et al., 1976; Miskhin et al., 1972; Ockner et al., 1982; Trulzch & Arias, 1981). There is reason to believe that intracellular movement of hydrophobic compounds will

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not require a transport protein in all cases. For example, relatively poor solubility in water and preferential partitioning into the lipid regions of intracellular membranes do not imply necessarily that the rate of hydration (eq 1) of a given hydrophobic compound will be slow as compared to catalytic events associated with its metabolism (Charlton et al., 1976; Doody et al., 1980).

$$\text{bilayer–ligand} + n\text{H}_2\text{O} \rightarrow \text{bilayer} + \text{ligand–(H}_2\text{O)}_n \quad (1)$$

The data for rates of reaction 1 are limited, and some of these data are for analogues of compounds of biologic interest. It is unknown whether data for compounds that do not occur in cells can be applied directly to considerations of the metabolism of the related compounds that occur naturally. In order to explore the possible pathways for movements of fatty acids within cells, we have measured the rates of eq 1 for fatty acids of varying chain length and unsaturation in unilamellar vesicles (ULV) of dimyristoylphosphatidylcholine. These measurements were made by using albumin as a "sink" for fatty acids released from ULV. The data, therefore, yield information on rates of hydration of fatty acids bound to albumin as well as lipid bilayers.

Materials and Methods

All fatty acids were obtained from P-L Biochemicals, Milwaukee, WI. Albumin (essentially fatty acid free from fraction V), dimyristoylphosphatidylcholine (DMPC), egg yolk lecithin (type III-E), and *N*-palmitoyldihydrolactocerebroside were purchased from Sigma. *Ricinius communis* agglutinin I (RCA 120 lectin) was from Vector Laboratories, Burlingame, CA. Organic solvents were redistilled over drying agents prior to use. All other chemicals were of the highest chemical purity available commercially and were used without further purification.

Fatty acids were prepared in argon-purged ethanol containing 0.001% butylated hydroxytoluene (BHT) as an antioxidant and stored at −20 °C under argon. Organic solvent were purged with argon and contained 0.001% BHT. The buffer used in all experiments was composed of 100 mM NaCl, 20 mM sodium phosphate, and 1 mM ethylenediaminetetraacetic acid (EDTA), pH 7.4, and purged with argon.

Analytical Methods. Lipid was extracted from aqueous solutions by the method of Folch et al. (1957). Phospholipid was assayed by the method of Morrison (1964). Protein was determined by the method of Lowry et al. (1951).

Preparation of Lipid Vesicles. Synthetic phosphatidylcholine (300 mg) was weighed out and dissolved in 3 mL of absolute ethanol, and the desired fatty acid (10 mM in absolute ethanol) was added and mixed. The solvent was removed under a stream of argon on a warming plate, and the dried lipid was placed under vacuum overnight. Buffer was added (20 mL) and the lipid sonicated under argon for 30 min with a Heat Systems sonicator employing a standard tip. The temperature was held above the phase transition temperature for the lipid being treated. Following sonication, the dispersed lipid vesicles were centrifuged for 20 min at 40 000 rpm in a 50 rotor (Beckman), and the supernatant was used directly for experiments. Vesicles were kept above their phase transition temperature, under argon, in order to prevent fusion.

Equilibrium Exchange Experiments. The equilibrium distribution of fatty acid between albumin and lipid vesicles was determined by a modification of the method of Backer & Dawidowicz (1979). Donor vesicles were prepared containing 10% glycolipid (by weight), 1 mol % fatty acid (^{3}H labeled), and a trace of [^{14}C]DPPC as nonexchangeable marker. The lipids were mixed in warm ethanol, dried under a stream of argon, and placed under vacuum overnight. The dried lipids then were dispersed in buffer by sonication. Vesicles and albumin were incubated at 30 or 37 °C for 30 min, which was sufficiently long to reach equilibrium. Following the incubation, a sample of the mixture was transferred to an Eppendax tube, and 0.3 mg of lectin was added to it. The mixture was stirred well and kept at 30 or 37 °C for 5–10 min. The samples were centrifuged; 200 μL of the supernatant was removed for determination of ^{3}H and ^{14}C. The percent of the fatty acid exchanged was calculated by using the expression

$$\text{percent exchanged} = \frac{H_s - C_s R_v}{H_t - C_s R_v} \times 100$$

For each aliquot from the incubation mixture, H_t is the total ^{3}H in the aliquot, H_s is the ^{3}H in the supernatant after removal of the donor, C_s is the ^{14}C in the supernatant after removal of the donor, and R_v is the ratio of the total ^{3}H and ^{14}C in the aliquot. Thus, the calculation of the amount of fatty acid exchanged takes into account the amount of unprecipitated vesicles in the supernatant.

Stopped-Flow Experiments. Solutions of albumin and liposomes containing fatty acids were mixed in a Durrum stopped-flow spectrophotometer interfaced with a Northstar Horizon computer. Control studies showed that the liposomes themselves had no effect on the fluorescence of albumin. The hardware and software for the data acquisition and manipulations were from On-Line Instrument Systems, Jefferson, GA. All solutions were buffered to pH 7.40 and contained 100 mM NaCl, 20 mM Na_2HPO_4, and 1 mM EDTA. Excitation was at 280 nm; fluorescence emission was measured with a 300–500-nm band-pass filter.

Assay of Acyl-CoA Ligase. Fatty acyl-CoA ligase was assayed according to Bar-Tana et al. (1971) modified in that no detergent was present. Fatty acid was added to the assay mixture as a complex with albumin. Reproducibility of the assay was ±5%.

Results

Transfer of Fatty Acids from ULV to Albumin Is Independent of the Concentration of Albumin. Partition coefficients for long-chain fatty acids added to a suspension of ULV in water are of the order of 10^6–10^8 in favor of the vesicle (Doody et al., 1980; Noy & Zakim, 1985a). The high avidity of fatty acids for lipid bilayers and the exceedingly low solubility of fatty acids in water make it difficult technically to measure rates of transfer of fatty acids from a lipid bilayer to water. To do so requires that the system be diluted so as to perturb the equilibrium distribution of fatty acids between the lipid and aqueous phases. The rate of approach to the new equilibrium distribution then is monitored. One must achieve a dilution sufficiently great that the approach to equilibrium can be monitored. It is obvious from the coefficients for partitioning of fatty acids between vesicles of phospholipids and water that the dilution factor for achieving a significant redistribution of fatty acid must be of the order of 10^4–10^6-fold. For the case that the rate of reaction 1 is rapid, i.e., half-times in the range of milliseconds to seconds, large enough dilutions followed by appropriate techniques for separating fatty acids in different phases cannot be accomplished. For example, the 10-fold dilution of the complex ULV–fatty acid that can be achieved within a stopped-flow mixing chamber would lead to the net movement from lipid to water of about 1 in 10^5–10^6 molecules of fatty acid as the system shifted from the old to the new point of equilibrium. Changes of this magnitude are too small to detect with optical methods. On the other hand,

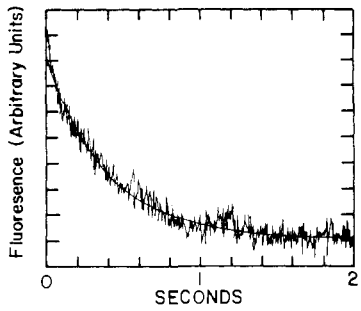

FIGURE 1: Time-dependent change in fluorescence of albumin after mixing albumin with unilamellar vesicles of DMPC containing 1 mol % myristate. A solution containing 15 μM bovine serum albumin was mixed with a solution of 5.5 mM DMPC in the form of unilamellar vesicles. The concentration of myristate in DMPC was 1 mol %. Measurements were made at 30 °C. Experimental conditions are given under Materials and Methods.

one can cause a large net movement of fatty acids out of the ULV by mixing the complex ULV–fatty acid with bovine albumin, which has two high-affinity binding sites for fatty acid per molecule of albumin (Spector, 1975). Moreover, since binding of fatty acids to albumin leads to quenching of the inherent fluorescence of this protein (Spector & John, 1968), the rate of quenching of fluorescence provides an optical method for following the transfer of fatty acids from bilayers to albumin (Figure 1).

Transfer of fatty acids from bilayers to albumin can occur theoretically by two different pathways. The fatty acid bound to lipids may become solvated prior to binding to albumin; or the transfer could occur according to reaction 2. If eq 2

$$\text{ULV–FA} + \text{albumin} \rightarrow \text{ULV} + \text{albumin–FA} \quad (2)$$

applies, the rate of transfer of fatty acids from bilayer to albumin will depend on the concentration of albumin.

When the concentration of the complex ULV–fatty acid was constant, the rate constant for quenching of the fluorescence of albumin decreased as the concentration of albumin added to the vesicles was increased (Figure 2, open circles). This apparently anomalous result can be understood according to the following analysis. The quenching of the inherent fluorescence of albumin depends on the fractions of its binding sites that are occupied. The total number of binding sites increases as the concentration of albumin increases. If albumin does not affect directly via reaction 2 the rate of release of fatty acids from the vesicles, then the fraction of fatty acid binding sites on albumin that are occupied at any given time will decrease with increasing concentrations of albumin, provided that the concentration of the complex ULV–fatty acid is constant. Data of the type depicted in Figure 1 must be used, therefore, to determine the absolute amounts of fatty acid bound to albumin at different time points along the time-dependent quench curve.

Albumin has several binding sites for long-chain fatty acids. On the basis of measurement of binding constants, two nonequivalent sites with high affinity for fatty acids and several different low-affinity sites have been identified (Spector, 1975). Titration with oleic acid of the two high-affinity binding sites (data not shown) indicated that binding at each of these sites led to incremental increases in the quenching of the fluorescence of albumin. The conditions of the stopped-flow experiments were such that about 2 mol of oleic acid was bound per mole of albumin at equilibrium after mixing of albumin with the complex ULV–oleic acid. It was possible to calculate in a straightforward manner, from the dependence of quenching on time, how much fatty acid was transferred from

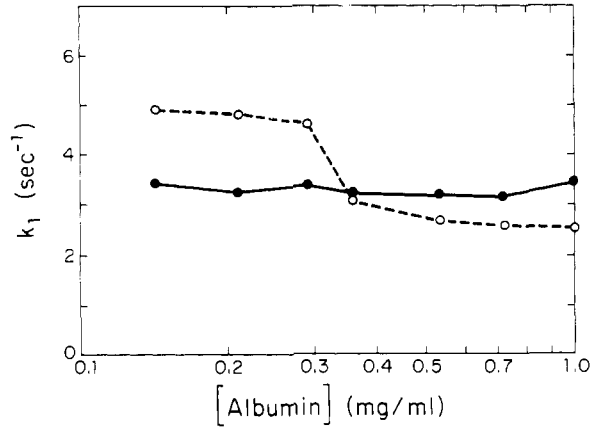

FIGURE 2: First-order rate constants for quenching of fluorescence of albumin after mixing with liposomes containing myristate. Conditions were the same as in Figure 1 and described under Materials and Methods except that the concentration of albumin was varied as indicated. Open circles represent the observed rate constants for quenching of fluorescence of the indicated amounts of albumin. Closed circles are rate constants for the time course of transfer of fatty acid from liposomes to albumin for variable concentrations of albumin. The data depicted by open circles were used to calculate the amounts of myristate transferred from DMPC to albumin at different time intervals. The method for making these calculations is given in the text. The amounts of myristate transferred for different times were used to calculate the actual first-order rate constants for transfer of myristate in the presence of variable amounts of albumin.

the bilayer to albumin at any given time. On the basis of the known relationship between quenching of fluorescence and fractional occupation of binding sites and the concentration of binding sites in each experiment, the amounts of fatty acid required to give the degree of quenching of fluorescence at each of several time points were calculated from the stopped-flow trace. The calculated amounts of fatty acid transferred at several time points were plotted as the log percent of fatty acids transferred at equilibrium. When the data were analyzed in this manner, the rate constant for transfer of fatty acids from ULV to albumin was independent of the concentration of albumin (Figure 2, closed circles). This result excludes the idea that transfer of fatty acids from ULV to albumin was mediated via collisions between these species. Instead, the data in Figure 3 suggest that transfer involves a slow, rate-determining solvation of fatty acid bound to vesicles followed by a rapid diffusion and uptake of fatty acid by albumin.

Rates of Hydration of Fatty Acids Bound to ULV as a Function of the Structure of the Fatty Acid. The transfer of fatty acids from ULV to albumin is an equilibrium system that can be described by eq 3 if one assumes that the steps of

$$\text{A} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \text{B} \quad (3)$$

diffusion through water and binding of fatty acids to vesicles or albumin are rapid as compared with the actual rates of hydration. It was not possible to force reaction 3 to completion, that is, to transfer to albumin all the fatty acids present initially within the vesicles of DMPC. As a result, the rate constants derived from the raw data contain k_1 and k_2 in them. Thus

$$-\frac{dA}{dt} = k_1(A_0 - A_t) - k_2A_t \quad (4)$$

At t = infinity, when the reaction is at equilibrium

$$k_1(A_0 - A_e) - k_2A_e = 0 \quad (5)$$

and

$$-\frac{dA}{dt} = (k_1 + k_2)(A_e - A_t) \quad (6)$$

Integration of eq 6 leads to

$$\ln (A_t - A_e) = (k_1 + k_2)t + C \quad (7)$$

Therefore, the rate constants calculated in Figure 2 from the raw data in Figure 1 are to be considered as k_{obsd}, where

$$k_{obsd} = k_1 + k_2 \quad (8)$$

The rate constant for reaction 1 is k_1, and k_2 is the rate constant for hydration of fatty acid bound to albumin. These rate constants are related by the equilibrium constant for the distribution of fatty acids between albumin and ULV (eq 9).

$$K_{eq} = k_1/k_2 \quad (9)$$

The equilibrium distribution of fatty acids between albumin and ULV is expressed as a partition coefficient. The unit of concentration of fatty acids in each phase is mole fraction. Since albumin contains binding sites for fatty acids that have variable affinity for these ligands, the partitioning of fatty acids between albumin and ULV will depend on the ratio of moles of fatty acid per mole of albumin for the system at equilibrium (Figure 3). However, over a range of amounts of fatty acid, albumin, and ULV, the distribution of fatty acids between albumin and ULV is a constant, when the moles of fatty acid per mole of albumin at equilibrium is close to 2. The partitioning of fatty acids between ULV and albumin depended on the chain length and degree of unsaturation of each fatty acid. Values for K_{eq} were 390 for myristate, 310 for palmitate, 75 for stearate, and 172 for oleate. Thus, if k_1 is considered to equal k_{obsd}, the error in k_1 is never more than about 1%. Of interest is that the partitioning of fatty acids between albumin and ULV depended on fatty acid structure in a manner that was different from the relationship between structure and affinity of albumin for fatty acids. The affinity of albumin for fatty acids increases with increasing chain length (Spector, 1975). However, the affinity of ULV for fatty acids, as a function of chain length, increases more rapidly per two-carbon addition vs. the increasing avidity of albumin for fatty acids of increasing chain length.

The data in Table I are values of k_1 for fatty acids bound to ULV of DMPC. The data show that the rate constant for solvation of fatty acid bound to ULV of DMPC depends on the nature of the fatty acid. This observation is important because it emphasizes the difficulty in extrapolating data obtained with nonphysiologic fatty acids to the situation of fatty acids of physiologic interest. The rate constants for reaction 1 show about a 10-fold decrease in k_1 for the addition of each two-carbon unit, as seen in the progression from myristate to palmitate to stearate. Introducing a double bond into the fatty acid structure at C(9)–C(10), e.g., palmitate vs. palmitoleic or stearate vs. oleate, increased the rate constant by about a factor of 7. Most important, the data indicate that the rate of eq 1 is quite rapid when fatty acids are the hydrophobic ligand. Whether or not the rate constant for solvation of stearic acid is slow enough to affect significantly rates of processes involving the metabolism of stearic acid in cells is unknown.

Effect of Temperature on k_1. Figure 4 contains a typical Arrhenius plot for the rate of solvation of oleate bound to vesicles of DMPC. All the data in Figure 4 were obtained above T_c for the ULV of DMPC. Table II contains values of E_a and calculated thermodynamic parameters for the activated state for solvation of several fatty acids bound to vesicles of DMPC. All the values for E_a were determined from plotting values of k_{obsd} as a function of T^{-1} absolute. The error in equating k_{obsd} with k_1 was small at all temperatures since measurements of the partitioning between bilayers and albumin of all fatty acids used in the range 30–40 °C confirmed that

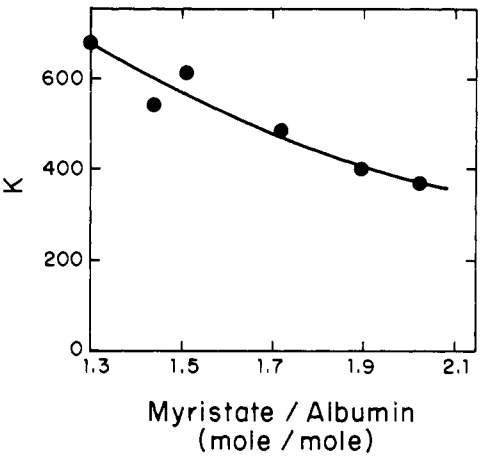

FIGURE 3: Partitioning at equilibrium of myristate between vesicles of DMPC and albumin. The distribution of myristate between DMPC and albumin was determined as described under Materials and Methods as a function of variable amounts of albumin but constant amounts of DMPC and myristate. The partition coefficient was calculated as the ratio of mole percent of myristate bound to albumin to the mole percent of myristate bound to DMPC. The initial concentration of myristate in DMPC was 1 mol %. Measurements were made at 30 °C in the buffer system described under Materials and Methods.

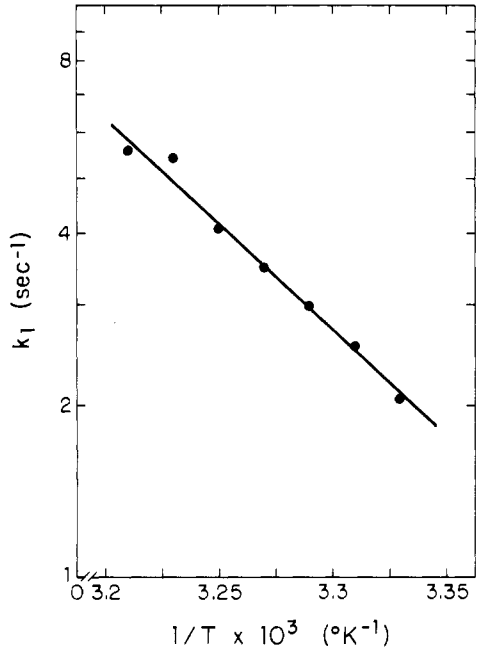

FIGURE 4: Effect of temperature on the rate of transfer of myristate from DMPC to albumin. Stopped-flow experiments were carried out as in Figure 1 and described under Materials and Methods at the indicated temperatures. Raw data for changes in quenching of fluorescence of albumin were used to calculate actual rates of change of the amount of fatty acid transferred from DMPC to albumin, as in Figure 3. At equilibrium, the albumin contained 2 mol of myristate per mole of albumin.

Table I: Rates of Solvation of Fatty Acids Bound to ULV at 30 °C[a]

fatty acid	k_1 (s)$^{-1}$	fatty acid	k_1 (s)$^{-1}$
myristate	35.2	stearate	0.46
palmitate	5.61	oleate	3.45
palmitoleate	39.2	elaidate	1.66

[a] The rate constant k_1 is the calculated rate of transfer of the given fatty acid from DMPC to albumin. The value k_1 was determined from raw data corrected according to the method described in the text and Figure 2 and the equilibrium distribution of the fatty acid between DMPC and albumin. All data were obtained under the condition that at equilibrium there were 2 mol of fatty acid bound per mole of albumin.

Table II: Thermodynamic Constants for Solvation of Fatty Acids Bound to Liposomes of DMPC[a]

fatty acid	E_a (kcal/mol)	ΔH^* (kcal/mol)	ΔG^* (kcal/mol)	ΔS^* (cal °C mol^{-1})
myristate	6.2	5.60	15.6	−33.0
palmitate	17.6	17.0	16.8	+0.4
palmitoleate	12.0	11.4	15.5	−13.7
stearate	17.4	16.8	18.2	−4.67
oleate	18.2	17.6	17.0	+1.97
elaidate	17.6	17.0	17.6	−2.02

[a] Rate constants used for calculations were for 30 °C. Activation energies (E_a) were determined from the slopes of Arrhenius plots, as in Figure 4. Values for ΔH^* were calculated from the expression $\Delta H^* = E_a - RT$. Values for ΔS^* were calculated from the expression $\Delta S^* = 2.30R \log (NhX/RT)$ in which $X = k_1/e^{-H/RT}$.

Table III: Rate Constants (k_2) for Solvation of Fatty Acids Bound to Albumin[a]

	temp			
	30 °C		37 °C	
fatty acid	k_2 (s^{-1})	$t_{1/2}$ (s)	k_2 (s^{-1})	$t_{1/2}$ (s)
myristate	0.098	7.11	0.120	5.78
palmitate	0.0186	37.2	0.035	19.8
stearate	0.0069	114.5	0.0098	70.9
oleate	0.020	34.8	0.0396	17.5

[a] The rate constant k_2 was determined by measurement of the first-order rate (k_{obsd}) at which the system DMPC–myristate + albumin approached equilibrium, as shown in Figures 1 and 3. The values of k_2 were calculated from the expressions $k_{obsd} = k_1 + k_2$ and K_{eq} (partition coefficient) $= k_1/k_2$.

the partition coefficient was never less than 75 (on a mole fraction basis) in favor of the albumin.

The data in Table II suggest that the fatty acids studied can be grouped into two classes. For one of these classes (myristate and palmitoleate), entropy changes from ground state to activated state are a major contributor to the energy of activation. For the other group (palmitate and C_{18} fatty acids), entropy changes are small whereas changes in enthalpy are the major contributor to the free energy of activation for solvation. It is remarkable that the variations of rates of solvation with temperature are nearly identical for the fatty acids most abundant as substrates for oxidation in mammals (palmitate, stearate, and oleate).

Rates of Solvation of Fatty Acids Bound to Albumin. The partitioning of fatty acids between ULV and albumin depends on the ratio at equilibrium of moles of fatty acids per mole of albumin. Partitioning favors albumin increasingly as the binding sites on albumin are less occupied with fatty acids (Figure 3). The most interesting results are for partition coefficients when the ratio of moles of fatty acids bound per mole of albumin is between 1 and 2 because this is the range for this ratio in vivo. Partition coefficients at 30 and 37 °C were measured for the condition that 2 mol of fatty acid was bound per mole of albumin when the system ULV, fatty acid, and albumin was at equilibrium. These values were used with the data in Table I to calculate k_2, the rates of solvation of fatty acids bound to albumin, which are shown in Table III for 30 and 37 °C. The striking aspect of these data is the slow rate at which fatty acids bound to albumin become solvated. The rates in Table III are considerably slower than data reported previously (Scheider, 1978; Svenson et al., 1974). In order to check the validity of the rates in Table III, we measured the rate of synthesis of stearoyl-CoA catalyzed by microsomal acyl-CoA ligase using stearate bound to albumin as the substrate. The time course for this reaction is shown in Figure 5. The time course shown by closed circles is for

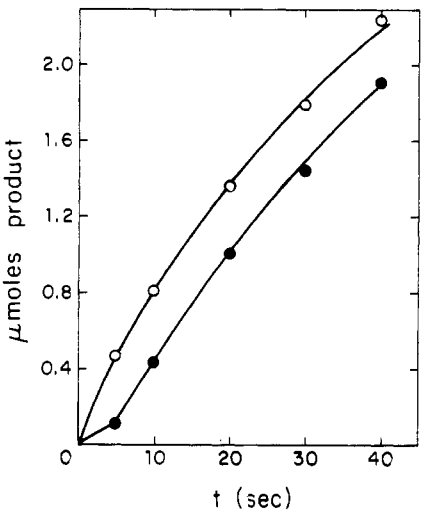

FIGURE 5: Time course for synthesis of stearoyl-CoA catalyzed by microsomal acyl-CoA ligase. The rate of synthesis of stearoyl-CoA was measured as described under Materials and Methods. Myristate was added to the assay as a complex with albumin (2 mol of myristate per mole of albumin). For data in open circles (○), the reaction was started by adding CoA to an otherwise complete reaction mixture. Microsomes and the complex albumin–myristate had been allowed to equilibrate for 15 min at 30 °C prior to starting the reaction. For data in closed circles (●), the reaction was started by adding the complex albumin–myristate to an otherwise complete reaction mixture.

synthesis of stearoyl-CoA under the condition that the reaction was started by addition of the complex albumin–stearate to microsomes, CoA, and ATP at 37 °C. Shown in open circles, for comparison, is the time course of synthesis of stearoyl-CoA when the complex albumin–stearate was added to microsomes and CoA at 37 °C and the reaction started 15 min later by addition of ATP. The data depicted with closed circles show a lag phase for about 5 s after initiation of the reaction followed by a steady-state rate that is maintained for about another 15–20 s. The reaction rate then declines. The presence and duration of this lag period and the shapes of the curves in Figure 5 were reproducible. The data depicted with open circles in Figure 5 show a rapid initial rate that declines continuously with time. The initial rate of activity for data in open circles is considerably greater than the steady-state rate for data in closed circles.

The data in Figure 5 were used to calculate a value for the rate of hydration of stearate bound to albumin. This calculation was based on the steady-state rate of utilization of stearate over 5–20 s of reaction. The rate over this interval was less that that for enzyme saturated with stearate (Noy & Zakim, 1985b) and thus would be expected to be sensitive to changes in the concentration of fatty acid within the microsomes. It has been shown previously that stearate within the microsomal membrane and not stearate in the aqueous phase is the proximate substrate for acyl-CoA ligase (Noy & Zakim, 1985a). The rate of synthesis of stearoyl-CoA was constant over the interval 5–20 s. Hence, the concentration of stearate in the microsomal membrane was not changing, and the moles of stearoyl-CoA synthesized between 5 and 20 s must equal the moles of stearate transferred from albumin to microsomes in this interval. The assay system at time zero (addition of albumin–stearate) contained 4.8 nmol of albumin and 9.6 nmol of fatty acid. The steady-state rate of synthesis of stearoyl-CoA was 0.066 nmol/s^{-1}. A rate constant for hydration of stearate bound to albumin of about 0.0066 s^{-1}, therefore, is sufficient to sustain the observed rate of synthesis of stearoyl-CoA. The value of k_2 determined from the stopped-flow experiment for equilibration of stearate between ULV

and albumin was 0.0098 s^{-1} (Table III).

Another way to calculate, from the data in Figure 5, the rate constant for hydration of stearate bound to albumin is to determine how much fatty acid needed to be transferred from albumin to microsomes in the interval 0–5 s to give the observed steady-state rate for the ligase-catalyzed reaction. The concentration of stearate in microsomes at $t = 5$ s in Figure 5 (closed circles) was determined from a double-reciprocal plot of rates of synthesis of stearoyl-CoA as a function of the concentration (mole fraction) of stearate in microsomal phospholipids at 37 °C. The relevant concentration of stearate was 0.0094 mol per mole of microsomal phospholipid. On the basis of the amount of microsomes in the assay in Figure 5 and that 0.12 nmol of stearate was metabolized between 0 and 5 s, 0.28 nmol of stearate was transferred from albumin to microsomes in the first 5 s of the assay depicted with open circles. The calculated rate constant needed for transferring this observed amount of stearate from albumin to microsomes in the interval 0–5 s is 0.0058. The data in Figure 5 therefore validate the value of k_2 determined from the stopped-flow data (Table III) for rates of hydration of stearate bound to albumin at a ratio of 2 mol of stearate per mole of albumin.

Discussion

The data presented suggest that fatty acids bound to biological membranes will move rapidly from membranes into the aqueous phase of the cell. Results from the work of Doody et al. (1980) lead to the same conclusion. The rate constant (k_1) for solvation of a pyrene-labeled fatty acid by Doody et al. (1980) was close to that for a naturally occurring fatty acid that is likely to perturb the environment of a bilayer of phospholipids with saturated acyl chains, as, for example, palmitoleic. On the other hand, the data reported in Table II indicate that the structure of the fatty acid studied has important affects on rates of eq 1. We have not measured as yet the effects of the composition of the bilayer on these rates, which it will be important to do. Since, however, the structure of the fatty acid has important effects on values of k_1, the data suggest that these rate constants also will depend to some extent on the acyl chains of the bilayer.

Doody et al. (1980) have pointed out already that the free energy of activation for hydration of pyrene-labeled fatty acid bound to ULV is considerably greater than the free energy change for the transfer of this fatty acid from the lipid bilayers to water. Thus, the transition state for the process is escape of the fatty acid from the membrane and its hydrated water or entry into water hydrated at the membrane surface and not entry into the bulk-phase water. This idea applies equally well to the hydration of the fatty acids examined in this paper. An interesting and important conclusion suggested by these data is that variations in the rates of reaction 1 for different hydrophobic compounds depend on the interactions between the hydrophobic compound and membrane lipids. For example, the rate of reaction 1 for cholesterol as compared with fatty acids is reported to be more than 500-fold slower (Giraud & Claret, 1979; Jonas & Maine, 1979; McLean & Phillips, 1981). There is considerable evidence that cholesterol is hydrogen bonded to the phosphates of membrane phospholipids in addition to interacting via van der Waals forces with the acyl chains of the phospholipids. The relatively slow rate of eq 1 for cholesterol, hence, may reflect the need to overcome the energy of this hydrogen bond. The thermodynamic data for solvation of fatty acids bound to ULV also are consonant with this idea. Rate constants (k_{obsd}) become smaller as the chain lengths of the fatty acid increases, and at the same time, enthalpies of activation become greater whereas the entropies of activation tend to become close to zero. Viewed in this way and in the context of the data presented above, the function of proteins that transport water-insoluble compounds through the cell is not so much to facilitate movement through water of compounds with limited solubility in water but rather to catalyze the release of these compounds from their attachments to membranes. It is important to consider, in this regard, that protein-facilitated transport between lipid vesicles has been demonstrated only for those compounds that become solvated from liposomes at rates that obviously can influence significantly the rates of subsequent metabolic steps, as, for example, cholesterol and phospholipids.

Implications of the Data for Mechanisms of Uptake of Fatty Acids by Tissues. Since the system studied is an equilibrium system, the data obtained yield estimates of the rates of solvation of fatty acids bound to albumin (k_2 in eq 8) as well as to ULV. The values of k_2 are significantly smaller vs. k_1, and values of $t_{1/2}$ for release of fatty acids from albumin containing about 2 mol of fatty acid/mol of albumin are on the order of seconds to minutes. The transport of fatty acids from storage sites in adipocytes to other tissues, and especially to the liver, occurs via binding to albumin (Havel et al., 1962). The data in Figure 3 and Table III reinforce the idea that albumin is suited ideally for this function because of its high affinity for fatty acids. That is, the rate of release of fatty acids of physiological interest from binding sites on albumin is considerably slower than the circulation time of blood. The tight binding of fatty acids to albumin ensures that fatty acids will not be released rapidly as blood flows in the great vessels and that the bulk of the fatty acids will be released where flow is sluggish, as in capillary beds and the sinusoids of the liver. It is interesting, in fact, that when the complex albumin–^{14}C-fatty acids is injected into animals, uptake of ^{14}C-fatty acids is greatest in the liver, which is the organ with the slowest flow and longest circulation time (Havel et al., 1962). Moreover, the rate of release of fatty acids from albumin depends on the moles of fatty acid bound per mole of albumin. Rates of release will increase significantly as this ratio increases from 0.5 to 2.0, which is the physiological range of binding of fatty acids to albumin.

Registry No. DMPC, 13699-48-4; stearoyl-CoA, 362-66-3; acyl-CoA ligase, 9013-18-7; myristic acid, 544-63-8; palmitic acid, 57-10-3; palmitoleic acid, 629-57-2; stearic acid, 57-11-4; oleic acid, 112-80-1; elaidic acid, 112-79-8.

References

Backer, J. M., & Dawidlowicz, E. A. (1979) *Biochim. Biophys. Acta 551*, 260–270.

Bar-Tana, J., Rose, G., & Shapiro, B. (1971) *Biochem. J. 122*, 353–362.

Bloj, B., & Zilversmit, D. B. (1977) *J. Biol. Chem. 252*, 1613–1619.

Catignani, G. L., & Bieri, J. G. (1977) *Biochim. Biophys. Acta 497*, 349–357.

Charlton, S. C., Olron, J. S., Hong, K.-Y., Pownall, H. J., Louie, D. D., & Smith, L. C. (1976) *J. Biol. Chem. 251*, 7952–7955.

Doody, M. C., Pownall, H. J., Kao, Y. J., & Smith, L. C. (1980) *Biochemistry 19*, 108–116.

Folch, J., Lus, H., & Stanley, G. H. S. (1957) *J. Biol. Chem. 226*, 497–509.

Giraud, F., & Claret, M. (1979) *FEBS Lett. 103*, 186–191.

Havel, R. J., Felts, J. M., & Van Duyne, M. (1962) *J. Lipid Res. 3*, 297–308.

Hellings, J. A., Kamp, H. H., Wirtz, K. W. A., & van Deenen, L. L. M. (1974) *Eur. J. Biochem. 47*, 601.

Jonas, A., & Maine, G. T. (1979) *Biochemistry 18*, 1722-1728.
Kamp, H. H., Wirtz, K. W. A., & van Deenen, L. L. M. (1973) *Biochim. Biophys. Acta 318*, 313-325.
Ketterer, B., Tipping, E., Hockney, J. F., & Beale, D. (1976) *Biochem. J. 155*, 511-521.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Rondall, R. J. (1951) *J. Biol. Chem. 193*, 265-275.
McLean, L. R., & Phillips, M. C. (1981) *Biochemistry 20*, 2893-2900.
Miskhin, S., Stein, L., Gatmaitan, Z., & Arias, I. M. (1972) *Biochem. Biophys. Res. Commun. 47*, 997-1003.
Morrison, W. R. (1964) *Anal. Biochem. 7*, 218-224.
Noland, B. J., Arebalo, R. E., Hansbury, E., & Scollan, T. J. (1980) *J. Biol. Chem. 255*, 4282-4289.
Noy, N., & Zakim, D. (1985a) *Biochemistry* (in press).
Noy, N., & Zakim, D. (1985b) *Biochim. Biophys. Acta 833*, 239-244.
Ockner, R. K., Manning, J. A., & Kane, J. (1982) *J. Biol. Chem. 257*, 7872-7878.
Scheider, W. (1978) *Biophys. J. 24*, 260-262.
Spector, A. A. (1975) *J. Lipid Res. 16*, 165-179.
Spector, A. A., & John, K. M. (1968) *Arch. Biochem. Biophys. 127*, 65-71.
Svenson, A., Holmer, E., & Anderson, L. O. (1974) *Biochim. Biophys. Acta 342*, 54-59.
Trulzch, D., & Arias, I. M. (1981) *Arch. Biochem. Biophys. 209*, 433-440.
Weisiger, R., Gollan, J., & Ockner, R. (1981) *Science (Washington, D.C.) 211*, 1048-1051.
Yamada, K., & Sasaki, T. (1982) *J. Biochem. (Tokyo) 92*, 457-464.

Sedimentation Equilibrium Measurements of the Intermediate-Size Tobacco Mosaic Virus Protein Polymers†

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ABSTRACT: Short-column sedimentation equilibrium methods have been applied for the first time to tobacco mosaic virus (TMV) protein (0.1 M ionic strength orthophosphate) at pH 6.5 and at pH 7.0 to estimate molecular weights. Previous sedimentation velocity experiments at pH 6.5, 20 °C have led to the conclusion that the major boundary with an $s^0_{20,w}$ value of 24.4 ± 0.1 S consists of a distribution of polymers which are mainly three-turn, 48-51-subunit helical rod aggregates. The directly measured z-average molecular weights together with sedimentation velocity data are entirely consistent with this assignment of a three-turn aggregate. Molecular weights have also been determined under two conditions where a large mass fraction of the protein sediments with an $s^0_{20,w}$ value of 20.3 ± 0.2 S. At pH 6.5, 6-8 °C, the aggregates in this boundary are metastable and correspond to 50-60% of the preparation. At pH 7.0, 20 °C at equilibrium, 65-75% of the protein sediments at 20.3 S. The 20.3S boundary is very similar under both conditions and is interpreted as being composed of a distribution of protein aggregates centered about 39 ± 2 subunits. This result is important in the interpretation of previous kinetic measurements of TMV self-assembly. The current view is that the 34-subunit structure of TMV protein, in the form of a cylindrical disk which is made up of two 17-subunit layers and has been characterized in single-crystal X-ray diffraction studies, plays a central role in the initial binding steps with RNA. The present results are not consistent with the view that there is a significant concentration of the TMV protein disk structure in solution under the usual conditions of TMV self-assembly.

The highly endothermic polymerization of tobacco mosaic virus protein (TMVP)[1] involves a single polypeptide species of 17 530 daltons and results in helical rods of varying length and similar shape to that of the native virus (TMV). At pH 7.0, 20 °C, 0.1 M ionic strength orthophosphate, the protein exists as a mixture of so-called 4S and 20S boundaries in an apparent weight ratio of approximately 30:70, respectively. The 20S boundary has been assigned as containing the obligatory nucleating species in TMV self-assembly (protein + RNA) (Butler & Klug, 1971). It has also been shown to act as the initiating material in the nucleation-controlled polymerization of TMVP to form helical viruslike rods (Schuster et al., 1979). This species has been referred to as a two-turn cylindrical disk [for a review, see Butler (1984) or Stubbs (1984)].

However, the only data which suggest a two-turn disk structure in low ionic strength solutions are from electron microscopy measurements (Crowther & Amos, 1971; Durham et al., 1971; Durham & Finch, 1972). The X-ray diffraction results of TMVP structure are derived from crystals in high ionic strength solutions where there is no doubt concerning the existence of a two-turn disk (17 subunits per turn) (Bloomer et al., 1978). Recent solution characterization of

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[1] Abbreviations: TMV, tobacco mosaic virus; TMVP, tobacco mosaic virus protein; A protein, TMVP in low states of aggregation, e.g., monomer through trimer, sedimenting at 3-5 S, depending upon the total concentration.

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