Different Mechanisms of Free Fatty Acid Flip-Flop and Dissociation Revealed by Temperature and Molecular Species Dependence of Transport across Lipid Vesicles*

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The mechanism of free fatty acid (FFA) transport across membranes is a subject of intense investigation. We have demonstrated recently that flip-flop is the rate-limiting step for transport of oleic acid across phospholipid vesicles (Cupp, D., Kampf, J. P., and Kleinfeld, A. M. (2004) Biochemistry 43, 4473–4481). To better understand the nature of the flip-flop barrier, we measured the temperature dependence of a series of saturated and monounsaturated FFA. We determined the rate constants for flip-flop and dissociation for small (SUV), large (LUV), and giant (GUV) unilamellar vesicles composed of egg phosphatidylcholine. For all FFA and vesicle types, dissociation was faster than flip-flop, and for all FFA, flip-flop and dissociation were faster in SUV than in LUV or GUV. Rate constants for both flip-flop and dissociation decreased exponentially with increasing FFA size. However, only the flip-flop rate constants increased significantly with temperature; the barrier to flip-flop was virtually entirely due to an enthalpic activation free energy. The barrier to dissociation was primarily entropic. Analysis in terms of a simple free volume (\(V_f\)) model revealed \(V_f\) values for flip-flop that ranged between \(\sim 12\) and 15 Å\(^3\), with larger values for SUV than for LUV or GUV. \(V_f\) values increased with temperature, and this temperature dependence generated the enthalpic barrier to flip-flop. The barrier for dissociation and its size dependence primarily reflect the aqueous solubility of FFA. These are the first results to distinguish the energetics of flip-flop and dissociation. This should lead to a better understanding of the mechanisms governing FFA transport across biological membranes.

Transport of long chain free fatty acids (FFA)\(^2\) across cell membranes is a necessary step for FFA utilization. Although considerable effort has been devoted to understanding the mechanism of cellular transport, there remains substantial disagreement as to whether transport is facilitated by membrane proteins or occurs by diffusion through the lipid phase (1–4). A protein-mediated mechanism would necessitate slow diffusion across the lipid phase, and therefore, understanding the mechanism of transport across simple lipid membranes has received considerable attention (5–13).

We have recently re-examined this issue and found that flip-flop is the rate-limiting step for transport of oleate across small (SUV), large (LUV), and giant (GUV) unilamellar vesicles and that dissociation is 5–10-fold faster than flip-flop (13). Previous conclusions (7, 11) that flip-flop was rapid and that dissociation was rate-limiting were based on incorrect interpretations of the results, primarily measurements of oleate influx into vesicles using oleate that was not complexed with serum albumin. We demonstrated previously that such measurements provide information about vesicle binding rather than flip-flop because the lipid bilayer is perturbed by exposure to high concentrations of unbound oleate when using uncomplexed oleate (13). In contrast, accurate information about flip-flop can be obtained by measuring influx using oleate complexed with bovine serum albumin (BSA) and/or measuring oleate efflux and dissociation from the vesicles (13).

The studies of Cupp et al. (13) revealed that flip-flop represents a major barrier to transport of oleate across the lipid bilayer and that this barrier increases with increasing vesicle diameter from SUV (\(\sim 250\) Å) to LUV and GUV (\(>1000\) Å). Thus, the lipid phase barrier to FFA flip-flop, at least in certain cell membranes, might be large enough so that the cell’s FFA metabolic requirements would necessitate a membrane protein transporter. In fact, we found a highly refractory lipid phase in our recent studies of FFA transport in adipocytes, where FFA transport was best described as mediated by an ATP-dependent transport pump (14, 15).

How the lipid phase can generate such large barriers is not known. The expectation of rapid flip-flop is based on the notion that flip-flop occurs by “Stokesian” diffusion through the hydrocarbon interior of the bilayer, a process equivalent to diffusion through an isotropic organic fluid. An isotropic solvent model is unlikely to provide an accurate representation of FFA flip-flop because of the anisotropic nature of the bilayer and the requirement for reorientation of the FFA within the bilayer. Moreover, diffusion of a solute through an isotropic solvent exhibits a relatively weak dependence (\(V^{-1/3}\)) on solute size, whereas studies of non-electrolyte solute permeation through lipid and red cell membranes reveal exponential dependences on solute size (16, 17). Lieb and Stein (16) have proposed that this steep size dependence reflects the polymer-like characteristics of the bilayer, in which diffusion proceeds by a “non-

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Stokesian” mechanism. In addition, the temperature dependence of diffusion through a Stokesian fluid should also be smaller than the dependence through a polymer (18). Thus, if the polymer-like nature of the bilayer affects FFA flip-flop, both the FFA size and temperature dependence of transport should be significantly different from those expected for a simple organic fluid.

A systematic investigation of the FFA size and temperature dependence of transport across lipid vesicles in which the flip-flop and dissociation steps were resolved has not been carried out previously. Previous results with selected FFA and temperatures need to be reconsidered because influx measurements were performed with uncomplexed FFA and because dissociation was not accurately separated from flip-flop (5, 7, 19). To develop a better understanding of the molecular basis for the steps involved in FFA transport across lipid membranes, we have, in this study, measured the temperature dependence for transport of a series of saturated and monounsaturated FFA of increasing size. Measurements were carried out in SUV, LUV, and GUV composed of egg phosphatidylcholine. The measurements allowed us to separate the FFA size and temperature dependence of the flip-flop and dissociation steps. The results indicate an exponential dependence of flip-flop and dissociation on FFA size. The barrier for flip-flop was dominated by large (15–20 kcal/mol) activation enthalpies and little or no entropy, whereas the barrier for dissociation was predominantly entropic. These results are inconsistent with a representation of the bilayer as a simple hydrocarbon fluid but suggest a more complex mechanism.

**EXPERIMENTAL PROCEDURES**

**Materials**—Egg phosphatidylcholine was purchased from Avanti Polar Lipids, Inc. (Alabaster, AL), and 1,α-dipalmitoyl-[choline,methyl-3H]phosphatidylcholine was from American Radiolabeled Chemicals, Inc. (St. Louis, MO). Sodium salts of the FFA were purchased from NuChek Prep (Elysian, MN), and stock solutions were prepared in water containing 4 mM NaOH (pH 11) and 50 μM butylated hydroxytoluene. Pyranine (8-hydroxypyrene-1,3,6-trisulfonic acid) was purchased from Molecular Probes (Eugene, OR). Acrylodan-labeled rat intestinal fatty acid-binding protein (ADIFAB) was prepared as described (20) and is available from FFA Sciences LLC (San Diego, CA). Fatty acid-free BSA was purchased from Sigma. The buffer used in FFA transport experiments contained 20 mM Hepes, 140 mM NaCl, and 5 mM KCl at pH 7.4 (buffer A), and both the FFA-BSA complexes and the vesicles were prepared in this buffer.

**Vesicle Preparation**—Vesicles were prepared as described recently (13). SUV, which are ~250 Å in diameter (21), were composed of egg phosphatidylcholine and sonicated in the presence of 0.5 or 2 mM pyranine in buffer A. LUV composed of egg phosphatidylcholine with 2 mM trapped pyranine or ADIFAB were prepared by extrusion and are ~1000 Å in diameter (22). GUV were prepared by dialysis of octyl β-glucopyranoside-solubilized egg phosphatidylcholine in which either 400 μM ADIFAB or 20 mM pyranine was also present and have diameters ~2000 Å (23). The last step for all three vesicle types was chromatography through Sephacryl S-1000 to separate free and trapped pyranine and/or ADIFAB. Vesicle phospholipid concentration was determined using the Elon method for total inorganic phosphate (24). Vesicle concentrations used in the stopped-flow experiments were between 5 and 200 μM. All stopped-flow concentrations refer to values in the mixing chamber, not in the syringe.

**Mechanisms of FFA Flip-Flop and Dissociation**—Complexes of FFA and BSA were prepared so that unbound FFA were buffered at defined values (13). Complexes of the unsaturated FFA were prepared by mixing aliquots of the sodium salt of the FFA from a 5 mM stock solution in water plus 4 mM NaOH at 37 °C with a 600 μM BSA solution in buffer A, also at 37 °C. For saturated FFA, the stock sodium salt solution was heated to between 55 and 70 °C, higher temperatures for longer chain FFA, before addition to the BSA solution at 37 °C. The concentration of free or unbound FFA was monitored several times during this titration using ADIFAB (25), and the final unbound FFA concentration ranged from 5 nM to 1.5 μM. A critical feature of the FFA-BSA complexes is that, at sufficiently high BSA concentrations, the unbound FFA concentration will not change upon addition of vesicles. The conditions for a well-buffered system depend upon unbound FFA, BSA, and vesicle concentrations and are determined by ensuring equal concentrations of unbound FFA in the complex and complex plus vesicles through direct measurement with ADIFAB. For a well-buffered system and sufficiently low unbound FFA concentrations, the influx time courses are well described by a single exponential. However, for a poorly buffered system, the influx time course reveals additional temporal components, both faster and slower than observed with the well-buffered system.

To measure FFA transfer between vesicles and BSA, SUV, LUV, and GUV were loaded with FFA by titrating a rapidly stirring solution of vesicles with aliquots of the sodium salt of the FFA. Titrating the vesicles with FFA was done by diluting a 50 mM sodium FFA solution in 4 mM NaOH (pH 11); raising the temperature to 37 °C; and distributing equal aliquots, waiting 2 min between aliquots, into a rapidly stirring solution. For efflux and transfer experiments, vesicle concentrations were between 25 and 100 μM, and the fraction of loaded FFA was between 10 and 20 mol %. Acceptor BSA was used at between 2 and 10 μM.

**Stopped-flow Fluorescence**—The kinetics of FFA movement were monitored by stopped-flow mixing with temporal resolution of <2 ms. Stopped-flow fluorescence was performed using a KinTek instrument, which allows two emission wavelengths to be detected simultaneously and in which equal volumes of 0.1-ml reagents were mixed at flow rates of ≥6 ml/s as described previously (13). All concentrations refer to the value in the mixing chamber. Tryptophan and pyranine fluorescence intensities were monitored by excitation at 290 and 445 nm, respectively, and observation of emission through 20-nm band-width filters at 343 or 505 nm, respectively. ADIFAB fluorescence was excited at 386 nm, and simultaneous emissions were measured at 432 and 505 nm. At least two separate preparations and >20 kinetic traces were generated for most experimental conditions.

**Analysis of FFA Kinetics**—Three different time courses were measured: 1) influx measurements in which the time course of
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FFA movement from the BSA donor to the vesicles was monitored by the change in fluorescence intensity of pyramine and/or ADIFAB trapped within the vesicle, 2) efflux measurements in which the time course of FFA movement from the vesicles to BSA in the extra-vesicle aqueous phase was monitored by trapped pyramine and/or ADIFAB fluorescence, and 3) transfer measurements in which FFA movement from the vesicles to extra-vesicle BSA was monitored by the change in BSA tryptophan fluorescence. The kinetic traces were fitted with multi-exponential functions to determine empirical rate constants for influx ($k_{in}$), efflux ($k_{out}$), and vesicle-to-protein transfer ($k_{trans}$). For the transfer measurements, we observed two rates: the $k_{trans}$ value is the faster (generally ~5–10-fold) of the two rate constants. We analyzed the transfer time course either allowing both components to vary or fixing the slow component to equal $k_{out}$. Both fits yielded virtually indistinguishable $\chi^2$ values and generally similar rate constants. We therefore used in most of our analyses $k_{trans}$ obtained with the slow component fixed as $k_{out}$.

To determine the intrinsic rate constants for flip-flop ($k_{in}$) and dissociation ($k_{off}$) that govern the time courses and to better understand their dependence on kinetic parameters, we used the analysis and simulation facilities of the programs MLAB (Civilized Software, Silver Spring, MD) and MACSYMA as described (13). This analysis accounts for the movement of FFA between BSA and vesicles and the movement of FFA across the bilayer as discussed by Cupp et al. (13). Back-transfer from BSA to the vesicles could contribute to the transfer kinetics, and therefore, the $k_{trans}$ values we report may be less than the $k_{off}$ values; however, in all cases, $k_{trans} > k_{out}$. We did not detect any significant change in $k_{trans}$ at 5–50 μM BSA for oleate (13) and in the selected cases examined in the present study (data not shown). We therefore conclude that, to a good approximation, $k_{in}$ and $k_{out}$ are independent of $k_{off}$.

As discussed by Cupp et al. (13), under conditions in which the FFA-BSA complexes buffer the unbound FFA, so that the unbound FFA concentration is the same in the presence or absence of vesicles, the determination of the inward flip-flop rate constant from the measured $k_{in}$ values is virtually independent of the BSA-bound FFA dissociation rate constant ($k_{off}$(BSA)). Our simulations (13) of the transport time courses demonstrated that this lack of dependence on $k_{off}$(BSA) results because, under buffered conditions, the fraction of BSA-bound FFA that is transferred to the vesicles is small (typically <2%), and therefore, the time needed to transfer is <2% of the $k_{off}$(BSA). Values for $k_{off}$(BSA) were obtained from our measurements of the dissociation of palmitate (C16:0), stearate (C18:0), oleate, and linoleate (C18:2Δ9Δ12) at 22 and 37 °C (26) and indicate that, in addition to oleate, $k_{in}$ should also be virtually independent of $k_{off}$(BSA) for these other FFA. Dissociation rate constants for the additional FFA used in the present study are not available, nor are values available for any of the FFA at all temperatures studied in this investigation. We have assumed, however, that because the unbound FFA was buffered for all FFA, binding and therefore $k_{in}$ are independent of $k_{off}$(BSA) for all FFA investigated.

Two features of our results provide experimental support for the accuracy of the flip-flop rates obtained from $k_{in}$. First, for all FFA and temperatures, $k_{in}$ and $k_{out}$ are quite similar, given the caveats concerning $k_{in}$ discussed under “Results.” Second, for the same FFA-BSA complexes, the $k_{in}$ values for SUV are ~10 times faster than for LUV or GUV. Because $k_{off}$(BSA) is independent of the vesicle type and $k_{in}$ does not involve BSA-vesicle collisions (13), the results indicate that $k_{in}$ is independent of $k_{off}$(BSA), at least for LUV and GUV.

Eyring Transition State Theory—The activation free energy ($\Delta G^\ddagger$), activation entropy ($\Delta S^\ddagger$), and activation enthalpy ($\Delta H^\ddagger$) were calculated using the Eyring rate theory as described previously (27). This analysis assumes that the thermodynamic model provides a reliable representation of the formation of the transition state and that the activation enthalpies and entropies are temperature-independent.

Free Volume Model—We have used the approach of Lieb and Stein (16) to extract free volume parameters that capture the FFA size dependence of our results. At each temperature, we analyzed the FFA molecular species dependence by expressing the rate constants for transport as in Equation 1,

$$k = k_o \exp(-V/V_f)$$

where $V_f$ is the average free volume for a given vesicle and temperature and $k_o$ is a pre-exponential factor. The molecular volume $V (\text{Å}^3)$ was estimated as $M_o \times 10^{27}/(N_a \cdot d^2 \cdot 1000)$, in which $M_o$ is the molecular weight of the FFA, $N_a$ is Avogadro’s number, and $d$ is the density of neat FFA and was adjusted ($d = 1.3$) to yield volumes consistent with those of Xiang and Anderson (18). At each temperature, the values of $k_{in}$ and $k_{out}$ as a function of the molecular volume for the saturated and monounsaturated series of FFA were used to determine $k_o$ and $V_f$ by least-squares fitting with Equation 1. Because the $k_o$ values generally showed little or no temperature dependence, we then repeated the analysis with the pre-exponential factor fixed to the value averaged over temperature and vesicle type, and the $V_f$ values from this analysis are reported here (Fig. 6).

RESULTS

Stopped-flow measurements were carried out to determine the three rate constants that define FFA transport across lipid vesicles: 1) transfer of FFA from BSA to the vesicle while monitoring trapped pyramine or ADIFAB ($k_{in}$), 2) transfer of FFA from the vesicle to BSA while monitoring trapped pyramine or ADIFAB ($k_{out}$), and 3) transfer of FFA from the vesicle to BSA while monitoring BSA tryptophan fluorescence ($k_{trans}$ and $k_{out}$). This approach was used in our previous study of oleate at 22 °C (13) and has now been extended to determine rate constants for 13 FFA using SUV, LUV, and GUV at temperatures between 15 and 37 °C.

A representative example of the measured time courses from which the three rate constants were determined (in this case, for the series of saturated FFA transported across LUV at 20 °C) is shown in Fig. 1. The results reveal a monotonic increase in all three rate constants with decreasing FFA chain length. These results also illustrate a feature common to all FFA studied and to the three types of lipid vesicles. The time course for the change in BSA tryptophan fluorescence resulting from transfer of FFA from the vesicles to BSA is
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FIGURE 1. Examples of the measured stopped-flow time courses for each of the three steps involved in FFA transport. The examples shown here are for the transport of the saturated FFA series across LUV measured at 20 °C. A, influx was measured by mixing FFA-BSA complexes with pyranine-containing LUV, and the traces show the decrease in trapped pyranine fluorescence corresponding to acidification of the vesicle interior. B, efflux was determined by loading pyranine-containing vesicles with the indicated FFA and then monitoring the increase in pyranine fluorescence upon mixing the vesicles with fatty acid-free BSA. C, transfer of FFA from the FFA-loaded vesicles to fatty acid-free BSA was monitored by the decrease in tryptophan fluorescence accompanying the increase in pyranine fluorescence upon mixing the vesicles with fatty acid-free BSA.

The rate constants determined from the time courses in Fig. 1 and are plotted as a function of carbon atoms per FFA for each of the three types of vesicles. Values of the natural log of the rate constants (k_{trns} and k_{out}) are shown, and the lines represent linear regressions.

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Composed of two components (k_{trns} and k_{out}), and in all cases, k_{trns} > k_{out}. This indicates that the rate constant for transfer from the vesicle surface to BSA (k_{trns}) is faster than k_{out} and, therefore, as found previously for oleate (13), flip-flop is the rate-limiting step for FFA transport across lipid vesicles.

The rate constants determined from the time courses in Fig. 1 as well as from the corresponding time courses for SUV and GUV at 20 °C are shown in Fig. 2 and Tables 1–3. For all three vesicle types, the three rate constants decreased exponentially with increasing chain length. The rate constants for SUV were larger by 10-fold compared with those for LUV and GUV, whereas LUV and GUV revealed similar rate constants. The results emphasize that k_{trns} > k_{out} and, in most cases, k_{trns} > k_{in} for the saturated FFA and all three vesicle types. Therefore, for all saturated FFA, dissociation from the vesicles is faster than flip-flop (k_{in} or k_{out}).

A similar variation of rate constants was observed for the series of monounsaturated FFA in SUV and LUV, except that the rate constants were ~10-fold lower for the monounsaturated FFA compared with the saturated FFA of the same chain length (Fig. 3 and Tables 1 and 2). Furthermore, for the same chain length (18 carbons), the rate constants increased exponentially with double bond number, from zero for stearate to 2 for linoleate (data not shown). Notably, k_{out} and, in most cases, k_{in} for these unsaturated FFA were significantly slower than dissociation. Taken together with the similar behavior of saturated FFA, these results confirm that flip-flop is the rate-limiting step for all long chain FFA and vesicles investigated in this study.

Transport rate constants were measured as a function of temperature and analyzed in terms of the Eyring theory to determine the activation free energies (∆G^{‡0}), enthalpies (ΔH^{‡0}), and entropies (TΔS^{‡0}) for the saturated FFA in the three vesicle types (Fig. 4). The ∆G^{‡0} increased linearly with chain length, consistent with a transport barrier that increases by ~4 kcal/mol with chain length from 14 to 19 carbons. The barriers for the three rate constants were ~1 kcal/mol larger in LUV and GUV compared with SUV; the larger vesicles revealed slower rate constants than SUV. Although the ∆G^{‡0} barriers increased uniformly for all three rate constants, the barrier for dissociation (∆G^{‡0}_{trns}) was smaller than the flip-flop barriers for all chain lengths.

The linear behavior of ∆G^{‡0} with FFA and its dependence on vesicle type are similar for the three transport steps, yet the mechanisms underlying flip-flop and dissociation are quite different. This is apparent from the differences in the ∆H^{‡0} and TΔS^{‡0} contributions to the barriers for flip-flop and dissociation (Fig. 4). For the influx and efflux steps, the enthalpic portion of the barrier dominated the free energy. In contrast, the free energy activation barrier for transfer was dominated by entropic factors.

Activation thermodynamic potentials for the monounsaturated series of FFA in SUV and LUV reveal a roughly similar behavior compared with the saturated FFA series (Fig. 5). However, there were significant differences between saturated and monounsaturated FFA. For influx and efflux, ∆G^{‡0} values were ~1.4 kcal/mol smaller than for the corresponding saturated FFA; this reflects the ~10-fold faster rate constants for the unsaturated FFA. On average, the smaller ∆G^{‡0} values for unsaturated FFA reflect smaller (~1 kcal/mol) enthalpic contributions. For transfer, the differences between saturated and monounsaturated ∆G^{‡0} values were smaller (~1 kcal/mol), and on average, the smaller ∆G^{‡0} for the monounsaturated FFA was due to a more favorable entropic contribution.

Because dissociation is virtually temperature-independent, whereas flip-flop increases exponentially with temperature, resolving the rate constants for dissociation from efflux becomes...
increasingly difficult with increasing temperatures, and at sufficiently high temperatures, the rate of flip-flop may exceed that of dissociation. This does not occur at temperatures ≤37 °C, although, as discussed below, k_{in} was occasionally ≥ k_{trns} for the shorter chain FFA probably because unbound FFA levels were too high.

We would expect rate constants for influx and efflux to be equal if flip-flop were symmetric. However, for all FFA and vesicles studied, k_{in} ≤ k_{trns}. This may reflect, in part, vesicle asymmetry because the k_{in} – k_{out} difference for SUV (~2-fold) was larger than for LUV and GUV (~60%). An additional contribution to this k_{in} – k_{out} difference is the FFA-induced perturbation of lipid vesicles as described previously for oleate transport (13). In the previous study, we showed that k_{in} increases with increasing unbound oleate concentrations, whereas k_{out} is much less sensitive to vesicle loading with FFA. Significant increases in k_{out} were observed only for [FFA]/[vesicle phospholipid] ≤ 0.2. We investigated the effect of increasing unbound FFA concentrations on k_{in} for selected FFA and found increases in k_{in} (data not shown), consistent with our previous oleate study. These effects were FFA type-dependent and appeared to be correlated with vesicle-water partition coefficients (K_v); for the same unbound FFA concentration, shorter chain and more unsaturated FFA were less perturbing. Because the pyranine response decreased with decreasing partition coefficient, higher unbound FFA concentrations were generally used for influx measurements for FFA with shorter chains and/or larger double bond numbers than for longer chain saturated FFA. For example, for myristate (C14:0), the unbound FFA concentration was 723 nM, whereas for nonadecanoate (C19:0), the unbound FFA concentration was 6 nM, but both generated similar equilibrium pH decreases.

**DISCUSSION**

The results of this study demonstrate that flip-flop is the rate-limiting step for FFA transport across lipid vesicles.

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**TABLE 1**

Rate constants for SUV

The estimated S.D. values from multiple determinations ranged between 10 and 30%.

| FFA   | 15°C | 20°C | 25°C | 30°C | 37°C |
|-------|------|------|------|------|------|
|       | k_{in} | k_{out} | k_{trns} | k_{in} | k_{out} | k_{trns} | k_{in} | k_{out} | k_{trns} | k_{in} | k_{out} | k_{trns} |
| C14:0 | 69     | 31    | 140   | 133   | 47    | 200     | 225    | 105    | 41     | 101   | 141    | 59     | 103 |
| C15:0 | 23     | 8     | 65    | 64    | 16    | 72      | 72     | 29     | 87     | 105   | 41     | 101    | 141   |
| C16:0 | 6      | 2     | 30    | 18    | 6     | 52      | 29     | 11     | 45     | 56    | 23     | 72     |
| C17:0 | 0.7    | 0.5   | 10    | 1.8   | 1.0   | 14      | 3.9    | 2.1    | 13     | 5.7   | 3.3    | 18     | 11.3  |
| C18:0 | 0.1    | 0.1   | 1.5   | 0.3   | 0.2   | 1.4     | 0.7    | 0.4    | 3.4    | 1.7   | 0.6    | 2.7    | 3.4   |
| C19:0 | 0.1    | 0.04  | 0.4   | 0.2   | 0.06  | 0.4     | 0.4    | 0.14   | 0.8    | 0.25  | 1.5    | 0.46   |
| C20:0 | 0.2    | 0.06  | 3.6   | 0.4   | 0.19  | 3.1     | 0.5    | 0.30   | 3.8    | 1.1   | 0.56   | 3.4    | 2.0   |
| C22:0 | 0.04   | 0.009 | 0.05  | 0.015 | 0.07  | 0.029   | 0.07   | 0.029  | 3.4    | 0.26  | 1.00   | 3.5    |

**TABLE 2**

Rate constants for LUV

Estimated S.D. values from multiple determinations ranged between 10 and 30%.

| FFA   | 15°C | 20°C | 25°C | 30°C | 37°C |
|-------|------|------|------|------|------|
|       | k_{in} | k_{out} | k_{trns} | k_{in} | k_{out} | k_{trns} | k_{in} | k_{out} | k_{trns} | k_{in} | k_{out} | k_{trns} |
| C14:0 | 16     | 6.6   | 106   | 19    | 10.1  | 150     | 34     | 13.4   | 148     | 46    | 19.7   | 120     | 82    |
| C15:0 | 4      | 1.7   | 38    | 6     | 2.7   | 35      | 7      | 4.8    | 43      | 13    | 6.9    | 52      | 27    |
| C16:0 | 0.9    | 0.4   | 9.3   | 1.4   | 0.6   | 10.7    | 2.4    | 1.0    | 7.6     | 3.6   | 1.5    | 6.1     | 6.8   |
| C17:0 | 0.3    | 0.07  | 0.4   | 0.12  | 0.2   | 2.3     | 0.7    | 0.2    | 3.5     | 1.3   | 0.4    | 3.2     | 2.8   |
| C18:0 | 0.04   | 0.05  | 0.04  | 0.04  | 0.57  | 0.08    | 0.07   | 0.50   | 0.22    | 0.14  | 0.54   | 0.21    |
| C16:1 | 5      | 4     | 46    | 11    | 4     | 50      | 21     | 11     | 80      | 25    | 20     | 109     | 52    |
| C17:1 | 1.4    | 1.6   | 17    | 2.1   | 2.4   | 23      | 5.7    | 5.3    | 38      | 8.3   | 7.9    | 71      | 18    |
| C18:1 | 0.6    | 0.4   | 5     | 1.4   | 0.7   | 1.8     | 1.8    | 0.8    | 4       | 3.5   | 1.7    | 7.5      |
| C19:1 | 0.05   | 0.04  | 1.1   | 0.06  | 0.09  | 2.0     | 0.27   | 0.16   | 1.1     | 0.48  | 0.26   | 1.1      |

**TABLE 3**

Rate constants for GUV

Estimated S.D. values from multiple determinations ranged between 10 and 30%.

| FFA   | 15°C | 20°C | 25°C | 30°C | 37°C |
|-------|------|------|------|------|------|
|       | k_{in} | k_{out} | k_{trns} | k_{in} | k_{out} | k_{trns} | k_{in} | k_{out} | k_{trns} | k_{in} | k_{out} | k_{trns} |
| C14:0 | 10     | 5     | 16    | 10    | 24    | 14      | 39     | 19     | 64     | 42    |
| C15:0 | 2      | 1     | 4     | 2     | 7     | 3       | 11     | 5      | 22     | 9     |
| C16:0 | 0.7    | 0.7   | 1.2   | 0.6   | 1.6   | 1.3     | 3.2    | 2      | 6.3    | 4     |
| C17:0 | 0.15   | 0.13  | 1.7   | 0.28  | 0.18  | 1.6     | 0.4    | 0.26   | 4.9     | 0.7   | 0.42   | 2.7      |
| C18:0 | 0.03   | 0.02  | 0.9   | 0.05  | 0.04  | 0.6     | 0.09   | 0.06   | 1.1     | 0.18  | 0.14   | 0.7       | 0.45  | 0.33 | 0.8    |
across LUV and GUV was ~10-fold slower than across SUV for all FFA investigated in this study. The barrier to FFA transport increased with FFA chain length for saturated and monounsaturated FFA and for the same chain length decreased with double bond number. The temperature dependence of FFA transport revealed that the barrier to flip-flop was primarily enthalpic, whereas that for dissociation from the vesicles was primarily entropic.

Previous Studies of FFA Molecular Size Dependence of Transport across Membranes—With the exception of our own work (13, 28), previous studies of long chain FFA have reported flip-flop rate constants that were faster than stopped-flow resolution and concluded that flip-flop was virtually independent of the molecular species of FFA (7, 11). As we demonstrated for oleate (13), the reports of extremely rapid flip-flop were incorrect because influx cannot be measured using FFA that is not complexed with albumin, as was done previously (7, 11). We also found that studies of FFA transfer from donor vesicle to acceptor BSA or acceptor vesicles in which changes inside the vesicles were measured determined efflux rather than dissociation (13), as assumed previously (5, 19). Indeed, the $k_{\text{out}}$ value for oleate we reported (13) and the “dissociation rate constant” of Zhang et al. (19) are in excellent agreement. For the five additional FFA (myristate, palmitate, margarate (C17:0), stearate, and linoleate) in the present study for which Zhang et al. (19) also reported values, our efflux rate constants are, on average, virtually identical to the dissociation rate constants of Zhang et al. (19), and both studies show similar trends with FFA molecular species.

Massey et al. (29) determined rate constants for the dissociation of a number of long chain FFA from SUV by monitoring albumin fluorescence as FFA transferred from donor vesicles, similar to the methods used in the present study. We noted previously in the case of oleate that our $k_{\text{trans}}$ and the $k_{\text{off}}$ of Massey et al. (29) were in good agreement (13), and we have found here that our dissociation rate constants for the three additional FFA (palmitate, stearate, and eicosenate (C20:1Δ13)) for which Massey et al. (29) also reported values agree, on average, to within a factor of 2 and display similar trends with FFA molecular species.

Membrane Permeability and Free Volume—In this study, we found that FFA flip-flop and dissociation are profoundly sensitive to the FFA type. Evidence of a size dependence for transport of molecules across membranes has been obtained previously in a number of studies of the permeability of non-electrolytes through lipid bilayers and membranes (16–18, 30, 31). These previous studies of small non-electro-
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**FIGURE 5.** Activation thermodynamic parameters for transport of monounsaturated FFA across SUV and LUV. The temperature dependence of $k_\text{in}$, $k_\text{out}$, and $k_\text{trans}$ was analyzed using the Eyring transition state theory to obtain values for $\Delta G^{\ddagger}$, $\Delta H^{\ddagger}$, and $T\Delta S^{\ddagger}$ for each unsaturated FFA and the two types of vesicles. The values are plotted against the number of carbon atoms per FFA.

Our transport results revealed exponential dependences on molecular size and temperature, suggesting that FFA transport may be better represented as diffusion through a polymer-like structure rather than through a Stokesian fluid. Diffusion across such a membrane would involve the creation of free volume (16) or free area (17, 18) and would reveal high activation energies. As discussed by Falck et al. (33), specific forms of free volume or free area models, at least for lateral diffusion in the bilayer, may be premature, and we therefore used the simple approach of Lieb and Stein (16) (see “Experimental Procedures”) to analyze our results.

The results of the free volume analysis of influx ($k_\text{in}$) and efflux ($k_\text{out}$) for the saturated and monounsaturated FFA series revealed monotonic increases in $V_f$ with temperature for all vesicle types (Fig. 6). Free volumes for influx and efflux averaged over all vesicles and temperatures yielded $13.5 \pm 1 \text{Å}^3$, remarkably similar to the value reported for red cells (16). In all cases, $V_f$ values for SUV were larger than for LUV or GUV. The increase with temperature in $V_f$ values obtained from the flip-flop rate constants is consistent with the enthalpic character of the Eyring activation free energy for flip-flop. By equating the Eyring and free volume equations for the rate constants, $\Delta H^{\ddagger}$

| Chain Length | SUV | LUV |
|--------------|-----|-----|
| $\Delta G^{\ddagger}$ (kcal/mol) | | |
| $\Delta H^{\ddagger}$ (kcal/mol) | | |
| $T\Delta S^{\ddagger}$ (kcal/mol) | | |

**FIGURE 6.** Free volume analysis of influx and efflux rate constants. The values of $V_f$ were determined at each temperature by fitting $k_\text{in}$ and $k_\text{out}$ for the saturated and monounsaturated FFA series with Equation 1 as described under “Experimental Procedures.” The analysis was carried out for all three types of vesicles (SUV, LUV, and GUV) for the saturated FFA series and for SUV and LUV for the monounsaturated FFA series. The natural log of the pre-exponential factor (ln($k_\text{in}$)) was fixed, as described under “Experimental Procedures,” at 25.5 and 25.0 for influx and efflux (upper panels), respectively, for the saturated FFA series and at 27 and 26 for the corresponding rate constants for the monounsaturated FFA series.
The slope of the $V_f$ temperature dependence and these calculated $\Delta H^{\circ}$ values are in good agreement with those obtained from the measured activation energies (Figs. 4 and 5). These results suggest that the rate-limiting enthalpic barrier reflects the energy needed to create free volume sufficient to allow FFA flip-flop between the outer and inner hemileaflets of the bilayer.

Although primarily entropic, the dissociation barrier includes a small enthalpic contribution (on average, 4 kcal/mol). This enthalpic contribution may be involved in the creation of free area needed to allow the FFA to dissociate from or insert into a hemileaflet of the bilayer. The origin of the entropic contribution to the dissociation barrier and its molecular size dependence are probably related to solvation of the FFA as discussed in more detail below.

**Vesicle Size Dependence**—Our results indicate that, for all FFA, both flip-flop and dissociation decrease with increasing vesicle size. Similar results were obtained in previous studies of cholesterol, anthroyloxyxystearate, and bilirubin dissociation and anthroyloxyxystearate and bilirubin flip-flop (27, 34–36). The similar LUV and GUV results we obtained are consistent with the detailed investigation of size dependence for bilirubin transfer, which revealed an asymptotic value for the transfer rate constant for vesicle diameters $>1000$ Å (36). The suggested origin of the size dependence of dissociation is a decrease in solute hydration or an increase in phospholipid packing with increasing vesicle size (36). Within the context of the free volume model, the vesicle size dependence can be accounted for by the larger $V_f$ for SUV compared with LUV or GUV. Whether free volume or some other characteristic of the different phospholipid order of SUV and LUV is an accurate description of how the bilayer affects flip-flop will require molecular models that are more refined than Equation 1.

**Model of FFA Transport through Lipid Bilayers**—The steps involved in FFA transport across the bilayer can be described with reference to Fig. 7B. We suggest that, from the aqueous phase (Step A), the FFA inserts tail first into the bilayer because $>98\%$ of the FFA is ionized at pH 7.4, and a carboxylate-first insertion would require an enthalpic activation energy of about 26 kcal/mol (37). However, dissociation (Figs. 4 and 5) and membrane partition (38) are largely entropic, and therefore, $k_{on}$ must also be largely entropic because $K_p = k_{on}/k_{off}$. Once in the bilayer, half of the membrane-bound FFA become protonated (39, 40).

As suggested by Xiang and Anderson (18), insertion of the FFA into the bilayer (Fig. 7B, Steps A to B) involves the creation of free area. We suggest that the energy ($\Delta H^{\circ}$) needed to generate this free area is small ($~4$ kcal/mol) because this area has to accommodate only the cross-section of the short axis of the FFA. Because this area is similar for all FFA, this step is probably not involved in the FFA size dependence of dissociation.

To flip across the bilayer, the FFA must, in addition to a translocation, undergo a 180° reorientation in which the polar carboxyl group reorients between the lipid-water interfaces on the two sides of the bilayer. (Flip-flop of the anionic form of the FFA is between 4 and 6 orders of magnitude slower than that of the protonated form (37, 41).) We speculate that, by slipping farther into the bilayer and rotating in a folded conformation (Fig. 7B, Steps C, D, and E), the FFA will undergo reorientation near the interface between the bilayer hemileaflets, where the membrane has substantially more disorder than near the head groups (42). The formation of the free volume and folding of the FFA may occur by torsional rotations along the acyl chains of the phospholipid and FFA. The ~10-fold larger flip-flop rates for monounsaturated FFA compared with saturated FFA
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may result because the cis-double bond itself provides a partial fold. The final step in the flip-flop process is the movement of the FFA from Steps E to F, the reverse of the Step B-to-D sequence.

Dissociation from the vesicle presumably occurs by creation of free area and ionization of the FFA, which likely dissociates as the anion. The FFA then becomes solvated in the aqueous phase, where it is at equilibrium (Fig. 7B, Steps B to A). Free energies for partition of oleate and palmitate are 10 kcal/mol and virtually entirely entropic (38), which, together with the $\Delta H^\circ$ of 4 kcal/mol, accounts for most of the $\Delta G^\circ$ of 15–17 kcal/mol for oleate and palmitate (Fig. 7A). That FFA solvation accounts for most of the dissociation barrier is consistent with findings based on short chain fluorescent FFA (43).

The observed size dependence of the dissociation rate constants reflects the size dependence of solvation. This conclusion is supported by comparing $\Delta G^\circ$ for dissociation with the equilibrium free energy change ($\Delta G^\circ$) for partitioning of FFA between water and heptane (44). The increase in $\Delta G^\circ$ with FFA carbon number (0.825 kcal/mol/carbon) is virtually identical to the value for $\Delta G^\circ$ ($0.820$) (Fig. 8). Furthermore, subtracting $\Delta G^\circ$ from $\Delta G^\circ$ yields a size-independent barrier of $-7$ kcal/mol. Although larger than the 4 kcal/mol attributed to free area formation, some of this disparity may be related to differences between heptane and the lipid bilayer. The faster dissociation of the monounsaturated FFA compared with the saturated FFA is also consistent with solubility because unsaturation increases the aqueous solubility of hydrocarbon chains (45).

Conclusion—Our results indicate that flip-flop is the rate-limiting step for FFA transport across lipid bilayer membranes and that the major barrier to flip-flop may be creation of a free volume large enough to accommodate the reorientation of an extended or partially folded FFA. The larger barrier for LUV and GUV and the correspondingly smaller $V_f$ compared with those for SUV suggest that interactions between the bilayer lipid components affect the barrier to flip-flop. Lipid components present in biological membranes may generate different barriers; our results in erythrocytes are consistent with a lipid phase and $V_f$ value similar to those of LUV (28), but our results in adipocytes suggest that the lipid phase is virtually impenetrable to flip-flop (14). This implies that at least certain biological membranes may require protein-mediated transporters to catalyze the flip-flop step. If $V_f$ formation is the barrier to flip-flop, vesicles with lipid compositions corresponding to those of erythrocytes and adipocytes may reveal different $V_f$ values. Studies to clarify this are now in progress.

Acknowledgment—We thank Jenny Chang for help with stopped-flow measurements.

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