Fatty Acid Flip-Flop in Phospholipid Bilayers Is Extremely Fast†

Frits Kamp,‡,§ David Zakim,§ Fengli Zhang,‡ Noa Noy,|| and James A. Hamilton*,‡

Department of Biophysics, Boston University School of Medicine, 80 East Concord Street, Boston, Massachusetts 02118, Division of Digestive Diseases, Department of Medicine, Cornell University Medical College, New York, New York 10021, and Division of Nutritional Sciences, Savage Hall, Cornell University, Ithaca, New York 14853-6301

Received February 20, 1995; Revised Manuscript Received June 26, 1995⊗

ABSTRACT: The rate of movement of fatty acids (FA) across phospholipid bilayers is an important consideration for their mechanism of transport across cell membranes but has not yet been measured. When FA move undirectionally across phospholipid bilayers, the rapid movement of un-ionized FA compared to ionized FA results in transport of protons. We have previously used this property to show that FA move spontaneously ("flip-flop") across the bilayer of small unilamellar vesicles within ~1 s (Kamp & Hamilton, 1992, 1993). This work extends the time resolution of this assay into the millisecond time range by use of stopped flow fluorometry. In small unilamellar vesicles (diameter, ~25 nm) at neutral pH, flip-flop of all fatty acids studied (lauric, myristic, palmitic, oleic, and stearic) was ≥80% complete within 5−10 ms. In large unilamellar vesicles (diameter, ~100 nm), the same fatty acids exhibited fast flip-flop but with a measureable rate ($t_{1/2}$ = 23 ± 12 ms). The calculated pseudounimolecular rate constant of the un-ionized FA (k_{FAH}) ~15 s^{-1}. There was no dependence of the flip-flop rate on the fatty acid chain length or structure. We also monitored the rate of desorption and transbilayer movement of (anthroyloxy)stearic acid in small unilamellar vesicles. Whereas previous studies suggested slow flip-flop of this FA analogue, the present studies suggest that (anthroyloxy)stearic acid flip-flops rapidly and that earlier studies did not truly measure the transbilayer movement step. These findings further support the view that proteins are not required for translocation of FA across cell membranes.

Passage of molecules across plasma membranes is a fundamental process in all cells, which have elaborated a variety of protein-based mechanisms for ensuring that substances of different sizes and chemical properties can either enter or leave under appropriate conditions. Selective proteinaceous pores exist in plasma membranes, for example, to allow uptake of small hydrophilic chemicals such as salts, sugars, and amino acids, which dissolve poorly within the hydrophobic regions of membranes. In addition, relatively large molecules are internalized by cells after the molecules bind to specific receptor proteins on the outer surface of the plasma membrane. Complex, protein-based mechanisms also exist for secretion of membrane-insoluble molecules. On the other hand, many molecules of biological and pharmacological significance have chemical properties and sufficiently small sizes to make them excellent solutes in the phospholipid regions of biological membranes. In vivo, such molecules will spontaneously distribute into cellular membranes. Whether passage of small hydrophobic compounds into the interior of cells needs to be facilitated by specialized proteins thus will depend on whether the rates at which they spontaneously transverse membranes and dissociate from membranes into the aqueous space of the cytosol are rapid enough to accommodate biological needs.

Long chain fatty acids (FA),[1] which have very limited solubility in water, partition avidly into phospholipid bilayers and natural membranes (Noy et al., 1986a,b; Daniels et al., 1985; Anel et al., 1993). The issue of how long chain FA transverse membranes is of special importance since, in addition to being the primary source of energy for many cells (Neely et al., 1974), they exert multiple effects on cellular physiology. For example, FA act as direct effectors of gene expression [e.g. Tugwood et al. (1992), Dreyer et al. (1992), and Zhang et al. (1993)], as activators of K^+ channels (Ordway et al., 1989), and as uncouplers of oxidative phosphorylation (Katiyar et al., 1991; Skulachev, 1991; Wrigglesworth et al., 1992). Modulation of energy metabolism and other cellular responses by long chain FA ultimately depends on the mechanism for entry of long chain FA into cells, which remains controversial. A long-standing theory (Spector et al., 1965) is that FA enter cells by passive diffusion through the plasma membranes. Recent support for this hypothesis includes observations that the rates at which FA spontaneously transverse lipid bilayers in small unilamellar vesicles and dissociate from them into water are very rapid (Daniels et al., 1985; Kamp & Hamilton, 1992, 1993). The rates in model membrane systems are faster than the rates of uptake of FA by target tissues such as the liver (Noy et al., 1986a, 1989; Cooper et al., 1987), suggesting that uptake does not require facilitation in vivo. Transbilayer movement of oleic acid in response to pH gradients imposed across lipid bilayers was inferred to be fast ($t_{1/2}$ < 1 s) in studies of Ca^{2+}-induced fusion of large unilamellar vesicles comprised of cardiolipin, dioleoylphosphatidylcholine, and cholesterol (Wilschut et al., 1992).

† This work was supported by NIH Grant HL26335.
* To whom correspondence should be addressed.
‡ Boston University School of Medicine.
§ Cornell University Medical College.
|| Cornell University.
⊗ Abstract published in *Advance ACS Abstracts,* September 1, 1995.

[1] Abbreviations: BSA, bovine serum albumin; FA, fatty acid; LUV, large unilamellar vesicles; NBD-PE, *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine; PC, egg phosphatidylcholine; SUV, small unilamellar vesicles; 12-AS, 12-(9-anthroyloxy)stearic acid.

0006-2960/95/0434-11928$09.00/0

The above studies of natural FA and other studies of modified FA [e.g. Doody et al. (1980)] have also suggested that the rate-limiting step for transfer of FA between lipid bilayers (or between bilayers and FA binding proteins) is the dissociation of these molecules from membranes and not the rate of flip-flop across bilayer leaflets. The most direct measurements of the transbilayer movement step, based on changes of pH inside vesicles, concluded that flip-flop proceeds with a half-time of less than 1 s in small unilamellar phospholipid vesicles, the time resolution of the experiments (Kamp & Hamilton, 1992, 1993). Interestingly, a recent study of movement of FA into pancreatic β-cells showed a slow time course for appearance of FA on the cytosolic side of the plasma membrane after addition of FA to the external buffer. However, these experiments did not measure solely the transmembrane movement step, and data suggested passive diffusion as the dominant mechanism for movement across the membrane (Hamilton et al., 1994).

On the other hand, it has been postulated that entry of long chain FA into cells is facilitated by a protein component in plasma membranes (Abumrad et al., 1981, 1993; Schwieterman et al., 1988; Storch et al., 1991). Although these proteins have never been isolated and then shown to facilitate FA movement through lipid membranes, the possible need for such proteins has been supported by biophysical studies of fluorescent (anthroyloxy) FA in model membranes (Storch & Kleinfeld, 1986; Kleinfeld & Storch, 1993). Data for transfer of anthroyloxy FA between phospholipid vesicles suggested that flip-flop of these FA across bilayers is slow and that transmembrane movement of naturally occurring FA likely required a transporter. The discrepancy between the results from studies carried out with naturally occurring vs anthroyloxy-labeled FA may indicate that the latter are a poor model for studies of uptake of the former.

The goals of the studies reported here were thus 2-fold: to attempt to measure the rates of flip-flop of FA across phospholipid bilayers by using a stopped flow technique and to verify whether significant differences can be found between the kinetic parameters of movement of physiological and anthroyloxy-labeled FA across lipid bilayers. Our results demonstrate that the rates of transbilayer flip-flip of both physiological and anthroyloxy-labeled FA are extremely fast. The data show further that removal of FA from both leaflets of unilamellar lipid bilayers proceeds by a single first-order process, which is the rate of hydration of membrane-bound FA:

$$\text{bilayer} - \text{FA} + \text{H}_2\text{O} \underset{k_{on}}{\overset{k_{off}}{\rightleftharpoons}} \text{bilayer} + \text{FA} - \text{H}_2\text{O} \quad (1)$$

MATERIALS AND METHODS

Chemicals. Egg phosphatidylcholine (PC) was from Avanti Polar Lipids, pyranin was from Kodak; *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine (NBD-PE) and 6-, 9-, and 12-(9-anthroyloxy)stearic acid (12-AS) were from Molecular Probes; fatty acid free bovine serum albumin (BSA), cholesterol, and lauric, myristic, palmitic, oleic, and stearic acids were from Sigma Chemicals.

Vesicle Preparation and Characterization. PC vesicles were made as before (Kamp & Hamilton, 1993). For cosonicated vesicles, the required amount of cholesterol (20 mol %), 12-AS (2 mol %), or NBD-PE (10 mol %) was mixed with PC dissolved in chloroform, prior to the evaporation of the solvents. When pyranin was trapped into the inner volume of the vesicles, 0.5 mM pyranin was present in the buffer that was mixed to the lipid film prior to the lipids being suspended (vortexed). The suspended lipid mixture (5 mg/mL of PC) was hydrated overnight at 4 °C. Small unilamellar vesicles (SUV) were made by sonicating the lipid suspension (5 mL) cooled in an ice−water bath for 20 min with a Heat Systems sonicator (power 3, pulsed mode 40% duty cycle). Large unilamellar vesicles (LUV) were made by freeze−thawing the lipid mixture 5 times, followed by extrusion (10 times) through two 100 nm polycarbonate filters stacked on top of each other using a Lipex extrusion apparatus (Olsen et al., 1979; Hope et al., 1985). All untrapped pyranin was removed by gel filtration (Kamp & Hamilton, 1993). The final PC concentration in samples used for data collection was determined by the Bartlett method (Bartlett, 1959). In all experiments with SUV, the buffer was 100 mM Hepes−KOH, pH 7.40. With LUV, either 100 or 25 mM Hepes−KOH, pH 7.40, buffer was used. Buffers were routinely filtered through an Amicon PM30 filter.

SUV preparations have previously been characterized by electron microscopy (Kamp & Hamilton, 1993). LUV preparations were examined by ^{31}P NMR to assess the size distribution and homogeneity of the preparations and by electron microscopy to determine the average particle size and to demonstrate the unilamellarity of the vesicles. ^{31}P NMR spectra were acquired at 121 MHz and at 30 °C on a Bruker AMX300 spectrometer with a 15 μs 90° pulse, 8K data points, a 2.2 s pulse interval, and composite ^{1}H decoupling. The spectra of LUV samples showed a broad (~500 Hz) Lorenzian peak characteristic of large vesicles; a narrow signal characteristic of SUV (Smith & Eikel, 1984) was not observed in these spectra. Transmission electron microscopy was performed on LUV samples without dilution or with dilution to ~0.5 mg/mL in deionized water as described for SUV previously (Kamp & Hamilton, 1993) and showed a predominance of unilamellar particles with $d \sim 1000$ nm. One LUV preparation was examined by vitreous ice electron microscopy, in which the sample does not have to be fixed and stained. This sample showed mostly large spherical unilamellar vesicles ($d \sim 1000$ Å), some with small unilamellar vesicles trapped in the center, and some multilamellar spherical vesicles. The ^{31}P NMR spectrum of this sample revealed predominantly a Lorenzian resonance of ~500 Hz and a small narrow component superimposed. The narrow signal represents small vesicular structures, possibly those trapped in the LUV, and also demonstrates that these small vesicles represent <5% of the total phospholipid.

Flip-Flop Assay Using Pyranin Fluorescence. PC vesicles with trapped pyranin were mixed in 2 mL of buffer at room temperature. Flip-flop of FA in SUV was monitored as before (Kamp & Hamilton, 1992), i.e. by adding FA dissolved in small aliquots of ethanol to the external medium with continuous mixing and following the decrease in internal pH as monitored by the pyranin fluorescence. Alternatively, flip-flop of the FA in the vesicles was determined by following the change in internal pH after the distribution of FA between inner and outer leaflet was altered by adjusting the external pH to pH 7.8 with KOH. The latter assay was also applied to vesicles containing cosonicated 12-AS. An ISS Gregg PC fluorometer was used for all experiments other than the stopped flow experiments. When the fluorescent probe was pyranin, excitation was at 455 nm and emission

was measured at 509 nm. The relation between pyranin fluorescence and internal pH was calibrated as described (Kamp & Hamilton, 1992). Under the chosen experimental conditions, pyranin displayed no photobleaching. 12-AS did not display any fluorescence at these wavelengths.

Stopped Flow Experiments. In control experiments, 100 μL of PC vesicles with trapped pyranin in buffer was mixed with an equal volume of distilled water. To measure FA flip-flop, the suspension of PC vesicles was mixed with an equal volume of distilled water containing 5−20 μM concentrations of FA (oleic, palmitic, or myristic acid) and 1 mM KOH (pH > 11) so that the FA were dissolved prior to mixing. The pH of the effluents, checked with a pH meter, was 7.40. Experiments were carried out with a High Tech stopped flow apparatus attached to a Fluorolog 2 SPEX fluorometer; the dead time was estimated at ~10 ms. The excitation wavelength was 455 nm and the emission wavelength 509 nm. Experiments were carried out at room temperature and in some cases at 2 °C.

Additional stopped flow experiments were performed with a KinTek, Inc., spectrophotometer operating at a constant temperature of 25 °C. The probe was excited at 445 nm, and the fluorescence intensity was monitored at 510 nm using a 1 in filter band-pass via a National Instruments analog/digital interface. We found this system to have a shorter dead time (<5 ms) but a lower sensitivity than the Specs fluorometer. For each sample, 10 successive runs were carried out and subsequently averaged for quantitative analysis. Fluorescence is reported as photon count (Specs) or volts (KinTek). Calibration of fluorescence vs pH was performed as with SUV. Analysis of fluorescence decays was performed by fitting the traces with single exponentials, since fitting with a double exponential led to only slight decreases in χ^2 (χ^2 for single exponential/χ^2 for double exponential was 0.97−1.15. χ^2 values for the single exponential fits were 2.8×10^{-3} to 3.6×10^{-4}.

Binding Assay for 12-(9-Anthroyloxy)stearic Acid. To PC vesicles with trapped pyranin in buffer was added an aliquot of 12-AS (dissolved in ethanol), and the combination was mixed. Binding of 12-AS was followed by monitoring of the fluorescence of 12-AS. The excitation wavelength was 383 nm, and emission was recorded at 455 nm. Pyranin did not display any fluorescence at these wavelengths.

FA Transfer Assay Using Pyranin Fluorescence. In these experiments, donor vesicles contained 2 mol % cosonicated 12-AS, and acceptor vesicles contained only PC. In one set of experiments, pyranin was trapped only in the donor vesicles, and in the other set, pyranin was trapped only in the acceptor vesicles. Donor and acceptor vesicles were mixed in 2 mL of buffer at room temperature.

FA Transfer Assay Using Quenching of Anthroyloxy Fluorescence by NBD. Donor vesicles contained 2 mol % 12-AS and acceptor vesicles 10 mol % NBD-PE, added by cosonication. No pyranin was present in either the donor or acceptor vesicles. In the control experiment, donor vesicles (13 μM PC) were mixed in 2 mL of buffer (at room temperature), and the 12-AS fluorescence was measured to establish the rate of photobleaching. In the transfer assay, donor vesicles (13 μM PC) were mixed with acceptor vesicles (170 μM PC), and the quenching of 12-AS fluorescence was measured.

RESULTS

Pyranin Assay for Flip-Flop of FA in SUV. Unidirectional transmembrane movement of FA generates a pH gradient across bilayers which can be measured by the quenching of a pH-sensitive, fluorescent molecule that is trapped on the inside of vesicles or cells (Kamp & Hamilton, 1992, 1993; Hamilton et al., 1994). Because of the low permeability of the membrane for ions (Kamp & Hamilton, 1993), these pH gradients can be maintained for minutes. Since the rate of hydration of FA bound to small unilamellar phospholipid vesicles (forward direction of reaction 1, $k_{off} = 5.6\ s^{-1}$ for palmitate; Daniels et al., 1985) and the partitioning constant for the distribution of FA between SUV and water are known ($K_{eq} = k_{on}/k_{off} = 5 \times 10^6$ for palmitate; Noy et al., 1986), one can show that the rate at which FA in water bind to membranes (reverse direction of reaction 1) is extremely fast ($k_{on} = 2.8 \times 10^7\ s^{-1}$ for palmitate). Therefore, when FA like oleic and palmitic acid are added to the outside of vesicles, the rate of any pH-induced change in the fluorescence of pyranin trapped within vesicles will, under appropriate mixing conditions, be limited by the rate of flip-flop of FA across the membrane of the vesicles.

Figure 1A shows the rapid acidification of the intravesicular volume of PC vesicles (SUV) following addition of palmitate to the external buffer, as previously reported (Kamp & Hamilton, 1992, 1993). This figure also shows an alternative method for measuring flip-flop of FA in bilayers, which is to alter the external pH after the FA have reached an equilibrium distribution between the inner and outer leaflets of the bilayer. This distribution was perturbed by addition of KOH to the external buffer. The consequent change in external pH from 7.40 to 7.87 resulted in an instantaneous increase in pyranin fluorescence, reflecting an increase in pH_{in}. This was expected because, in response to an increase in external pH, the fraction of ionized FA in the outer leaflet will increase (Hope & Cullis, 1987). Subsequently, some un-ionized FA will move rapidly from the inner leaflet to restore equilibration of un-ionized FA between the inner and outer leaflets. Re-equilibration of the FA ionization on the inner leaflet will remove some H^+ from the aqueous compartment. As expected, the magnitude of this change is much smaller than the initial pH change after addition of palmitic acid to the external buffer. A control experiment with no added FA showed no change in fluorescence upon similar alteration of the external pH (not shown). Thus, there is no binding of pyranin to the outer leaflet of the membrane.

Stopped Flow Experiments with SUV. In Figure 1A, and in our prior studies, the pH-dependent changes in the fluorescence of pyranin in SUV were completed within the mixing time of the experiments, e.g. 1−3 s (Kamp & Hamilton, 1992, 1993). This time resolution yields only an upper limit (~1 s) to the true rate of flip-flop. To improve on the resolution of this method, we attempted to measure flip-flop rates on the time scale of milliseconds by stopped flow fluorometry. To this end, buffered aliquots of SUV with trapped pyranin were first mixed rapidly with equal volumes of distilled water to establish the fluorescence level of the vesicles upon dilution in the absence of any FA. The upper traces in Figure 1B,C represent these control experiments. Although there is noise in the traces because of light scattering by the vesicles, the level of fluorescence was stable across the time course of the recording interval. When

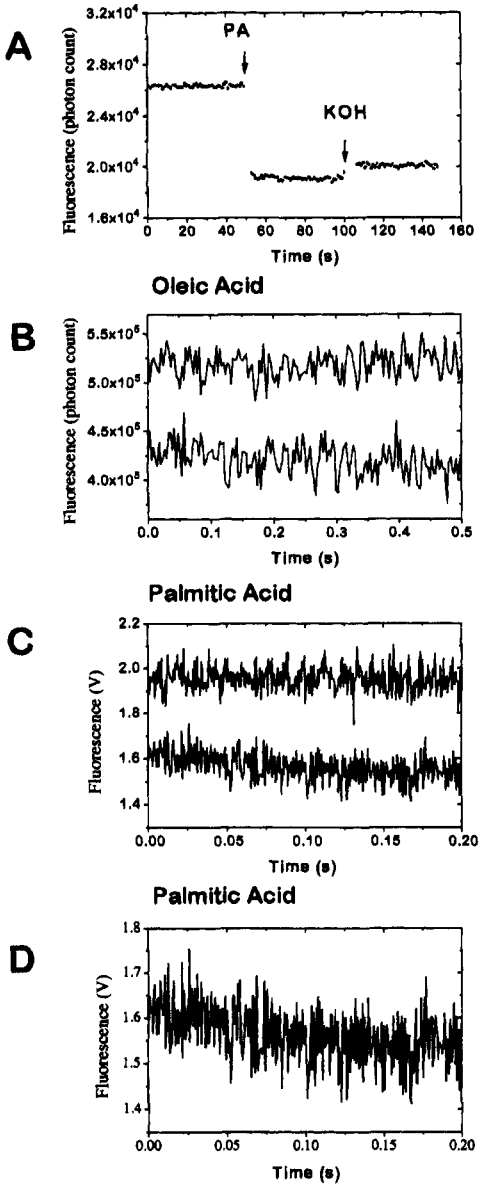

FIGURE 1: Pyranin assay for flip-flop of FA in SUV. (A) Addition of 20 nmol of palmitic acid (PA; 11 mol % relative to PC) to PC vesicles (90 μM PC) with trapped pyranin in 2 mL of buffer (100 mM Hepes) at pH 7.40. The large decrease in fluorescence, corresponding to a drop in pH_{in} of ~0.5 pH unit, is caused by flip-flop of un-ionized FA to the inner leaflet of the vesicle. Subsequent addition of KOH, which increased the external pH to 7.83, caused a small increase in fluorescence, reflecting redistribution of FA to the outer leaflet in response to the altered pH gradient. (B) Stopped flow experiment to measure FA flip-flop on a millisecond time scale. The upper trace represents the control experiment, i.e. mixing of the vesicles (570 μM PC) with distilled water (sampling time, 3 ms); the lower trace represents mixing with potassium oleate (2 mol % relative to PC; sampling time, 2 ms). The decreased level of fluorescence indicates flip-flop of un-ionized FA to the inner leaflet of the vesicle. This was essentially complete with the dead time of mixing (10 ms). (C) Stopped flow experiment as in part B with palmitic acid. The upper trace represents the control experiment. The lower trace represents mixing of the vesicles with 7 mol % potassium palmitate. Sampling time was 0.5 ms; dead time was 5 ms. The traces each represent the average of 10 trials. (D) Vertical expansion of the lower trace of part C, showing small decay in the first 50−100 ms.

vesicles were mixed in the stopped flow apparatus with an equal volume of a solution containing either potassium oleate or palmitate, the level of fluorescence was lower (lower traces in Figure 1B,C). As seen most clearly in the expanded bottom trace of Figure 1C, there was a small change in fluorescence during the time of recording (Figure 1D). However, the fluorescence had decreased to 80% of the equilibrium value within the mixing time (≤5 ms). This result means that FA added to the outside of the vesicles equilibrated rapidly across the bilayer, with concomitant delivery of protons into the internal volume (Kamp & Hamilton, 1992, 1993). Identical results were obtained with potassium laurate, myristate, and stearate (data not shown). We conclude, therefore, that long chain FA flip-flop across phospholipid bilayers in SUV with a half-time that is faster than 5 ms. The small decay in fluorescence suggests that flip-flop occurs just below the threshold of measurement (5−10 ms) rather than in the microsecond time range.

We attempted to slow the rate of flip-flop in SUV and possibly make it measurable by lowering the temperature of the experiments to 2 °C. Addition of oleate or palmitate in a stopped flow experiment at 2 °C gave results identical with those in Figure 1B,C; i.e. the system reached an equilibrium distribution (>80%) of FA between inner and outer halves of the bilayer within the mixing time of the instrument (data not shown). We also investigated whether the presence of cholesterol would decrease the rate of flip-flop to a measurable rate. However, equilibration of FA between inner and outer halves of the bilayer also occurred within the mixing time of the stopped flow instrument when vesicles contained 20 mol % cholesterol, even when the temperature was lowered to 2 °C (data not shown).

Flip-Flop in Large Unilamellar Vesicles. The curvature of plasma membranes is generally lower than the curvature of SUV, and it is possible that lower curvature decreases the rate of spontaneous transbilayer movement of FA. Therefore, we examined the effect of curvature on the rate of flip-flop by repeating the pyranin assay with LUV of 100 nm diameter. When FA was added to a stirred cuvette containing LUV, instant acidification (within 2 s) of the internal volume was observed, which was reversed when FA was subsequently removed from the vesicles by the addition of BSA, as found for SUV. The experiment was repeated with various amounts of FA, and the decreases in pH plotted against the mole % of FA in PC closely matched the predicted behavior (Figure 2A). The much larger volume/surface ratio for LUV compared to that for SUV (LUV = 4.0 μL/μmol of PC and SUV = 0.3 μL/μmol of PC, based on diameters of 100 and 25 nm, respectively) had two important effects on the assay conditions. First, the concentration of LUV to achieve fluorescence levels similar to SUV (about 0.05 mM PC compared to 0.5 mM PC) was about 10 times lower. Second, higher amounts of FA relative to PC were needed to observe similar pH changes in the internal volume. The latter effect is readily predicted from the theoretical curve shown in Figure 2A, calculated as for SUV (Kamp & Hamilton, 1993) but using a diameter of 100 nm for LUV.

Figure 2B displays a stopped flow experiment with LUV performed with the same protocol as for SUV (Figure 1B,C). As opposed to the findings with SUV, upon addition of K^+ palmitate, there was a single exponential decay with a measurable $t_{1/2}$ (35 ms). However, the amount of palmitate required to produce this change in fluorescence (26 mol %) was much higher than that for SUV. Similar results were obtained with several preparations of LUV with palmitate and oleate.

The preceding experiments with both LUV and SUV were done in 100 mM Hepes buffer, which provides a strong

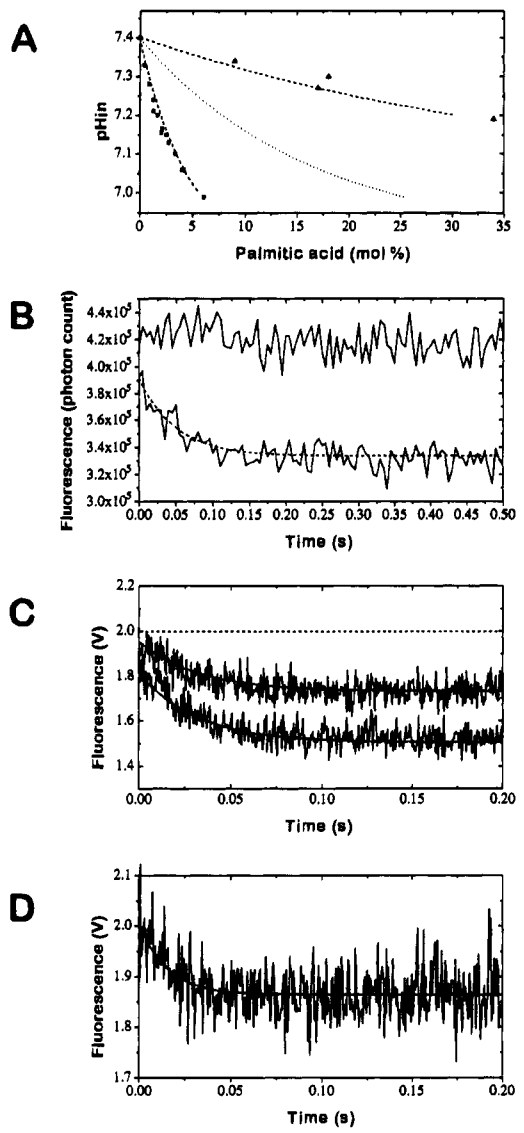

FIGURE 2: Pyranin assay for flip-flip in LUV. (A) Changes in internal pH upon addition of increasing amounts of FA to SUV (squares) and LUV (triangles) made in 100 mM Hepes buffer, pH 7.4. The dashed lines represent the calculated changes in pH_{in}. When LUV were made in 25 mM Hepes, the pH changes were about 4 times larger (dotted line). (B) Stopped flow experiment. The upper trace represents the control experiment, in which a suspension of LUV (56 M PC in 100 mM Hepes) was first mixed with an equal volume of distilled water. The lower trace represents a single experiment in which K^+ palmitate (26 mol % relative to PC) was added to the LUV suspension. The decrease in fluorescence was fit to a single exponential (dashed line) and corresponded to a pH change of 0.2 unit. Sampling time was 2 ms; dead time was 10 ms. The $t_{1/2}$ was 35 ms, and the calculated k_{FAH} pseudounimolecular rate constant = 13 s^{-1}. (C) Stopped flow experiments. LUV were prepared in 25 mM Hepes, pH 7.4. The dashed line represents the control experiment as above. LUV (150 μM PC) were mixed with 7 mol % (upper trace) and 16 mol % K^+ palmitate (lower trace). The illustrated traces represent the average of 10 trials. Sampling time was 0.5 ms; dead time was 5 ms. The total decrease in pH_{in} was 0.17 unit (7% palmitate) and 0.30 unit (16% palmitate). The solid lines are the single exponential fits. The $t_{1/2}$ was 22 ms for both 7 and 16 mol % palmitate. The calculated k_{FAH} values were 16 and 13 s^{-1}, respectively. (D) Stopped flow experiment as in part C, but with 7 mol % K^+ laurate. The total decrease in pH was about 0.1 unit. The smaller decrease relative to palmitate is expected because not all of the laurate binds to the vesicle (Kamp & Hamilton, 1993). The dotted line is the single exponential fit ($t_{1/2}$ = 11 ms).

buffering capacity against the small amount of H^+ transported by the FA and gives a good working range for changes in pH in SUV but limits the sensitivity of the assay in LUV. As shown in Figure 2A, a buffer comprised of 25 mM Hepes, pH 7.4, is predicted to give a higher sensitivity for measurements of flip-flop in LUV. We therefore performed additional stopped flow experiments with LUV prepared in 25 mM Hepes, pH 7.4, to monitor relatively low proportions of FA in the vesicles. As illustrated for palmitate (Figure 2C), significant decreases in fluorescence were seen with lower levels of FA (such as 7 and 16 mol % illustrated). The extent of the decreases in pH depended on the amount of palmitate added. Experiments with LUV in 25 mM Hepes were repeated with different amounts of laurate, myristate, and stearate. In all experiments with LUV in 25 mM Hepes, we observed an average $t_{1/2}$ of 18 ± 6 ms for flip-flop. The accuracy of this determination was limited by the signal to noise of the fluorescence traces, but there was no significant dependence of $t_{1/2}$ on the amount of FA or the FA chain length.

The observed initial rate of acidification in the above experiments depends on the experimental conditions chosen, such as the amount of FA, the internal volume of the vesicles, the buffering strength, and the pH_{in} and pH_{out}. The pseudounimolecular rate constant of flip-flop of un-ionized FA (k_{FAH}) does not depend on the above experimental conditions and can be calculated from the initial rates of acidification of the fluorescence traces (e.g., Figure 2B,C) with the use of equation 2 derived in the appendix of Kamp et al. (1993). Thus,

$$k_{FAH} = -(d(pH_{in})/dt)^{t=0}(B_{in}/N)(1 + 10^{(pK_a - pH_{in})})(1 + 10^{(pH_{out} - pK_a)}) \quad (2)$$

In this equation, $(d(pH_{in})/dt)^{t=0}$ is the initial rate of acidification upon addition of FA, B_{in} is the internal buffering capacitance [B_{in} = 200 nmol of H^+/(micromole of PC per pH unit) for LUV with a diameter of 100 nm and 100 mM Hepes−KOH, pH 7.4; when 25 mM Hepes is trapped, B_{in} = 50 nmol of H^+/(micromole of PC per pH unit)], N is the total amount of added FA (nanomoles of FA per micromole of PC), pK_a = 7.6 for FA (Hamilton, 1995), and pH_{in} = pH_{out} = 7.4 at t = 0. When a single exponential fit is applied to the traces of Figure 2B,C, the initial rate of acidification is related to the $t_{1/2}$ of the fit as follows:

$$(d(pH_{in})/dt)^{t=0} = (0.69/t_{1/2})(pH_{in}[t = 0] - pH_{in}[t = \infty]) \quad (3)$$

Using the results from Figure 2B,C (upper and lower traces), the calculated k_{FAH} values were 13, 16, and 13 s^{-1}, respectively. Similar values (within a factor of 2) were found for lauric, myristic, and stearic acids. Because of the signal to noise ratios of the fluorescence data, we cannot distinguish rate constants that are within a factor of about 2. We conclude that flip-flop of FA in LUV occurs in the time range of milliseconds and does not depend on the chain length of the FA.

Flip-Flop of Anthroyloxy-Labeled FA. Anthroyloxy-labeled FA have been used to study movement of FA across lipid bilayers because of the fluorescence of the anthroyloxy group, although it is not clear whether these derivatized FA are representative of native FA (Storch & Kleinfeld, 1986; Kleinfeld & Storch, 1993). In previous experiments, we found that addition of 2 mol % 12-(anthroyloxy)stearic acid

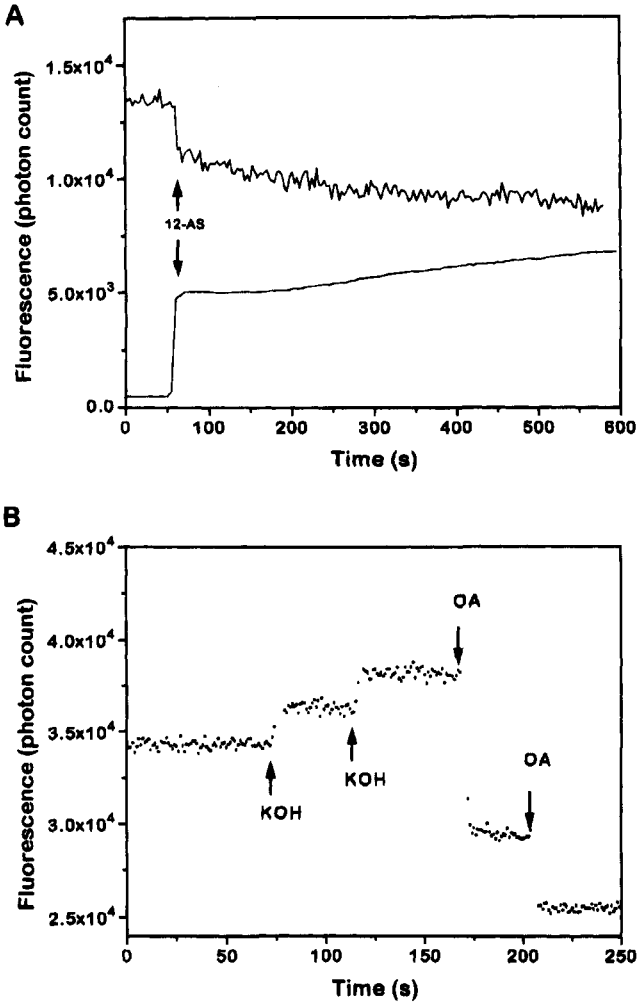

FIGURE 3: Pyranin assay for flip-flop of anthroyloxy FA (12-AS). (A) Addition of 0.2 mol % 12-AS to PC vesicles (100 μM PC) with trapped pyranin in 2 mL of buffer. Upper trace: pyranin fluorescence. Lower trace: 12-AS fluorescence. The scale of the 12-AS fluorescence was reduced 100-fold. Both experiments indicate incomplete instantaneous binding of 12-AS to vesicles. (B) PC vesicles (300 μM PC) with trapped pyranin and 2 mol % cosonicated 12-AS in 2 mL of buffer at pH 7.40. First, two aliquots of KOH (5 μL of 1 N KOH) were added, increasing the external pH to 7.87. Subsequently, 30 nmol of oleic acid (15 mol % relative to PC) was added, followed by a second aliquot of 30 nmol of oleic acid.

(12-AS) to vesicles containing pyranin caused a much slower decrease in internal pH than did native FA. We concluded that flip-flop of 12-AS was much slower than that of native FA on the time scale suggested by Kleinfeld and Storch (1993). Careful re-examination of our prior data revealed, however, that the kinetics follow neither a single or a biexponential function, which suggests that we previously measured a process more complicated than we appreciated. Moreover, other FA with covalently attached bulky groups show fast flip-flop by the pyranin assay (Kamp & Hamilton, 1993).

To gain a better insight into the mechanism of flip-flop, we investigated (anthroyloxy)stearic acid with labels in the 6, 9, or 12 positions. Whereas high concentrations of 12-AS, as previously used (Kamp & Hamilton, 1993), resulted in a slow decrease in the fluorescence of pyranin in vesicles, low concentrations showed an instantaneous drop in fluorescence of pyranin followed by a much slower rate of decline (Figure 3A, upper trace). The relative decline in total fluorescence of the extremely fast component was larger at lower concentrations of 12-AS. These results can be explained by instant precipitation of some of the 12-AS added to the buffer solution. The amount that stayed in solution immediately entered vesicles and traversed the bilayers to account for the rapid phase of fluorescence quenching. The fraction of the anthroyloxy FA that precipitated then dissolved slowly into the water phase and equilibrated between the water and the vesicles, accounting for the slow phase of quenching of the fluorescence of pyranin. Similar results were obtained for 6-AS and 9-AS.

These ideas were supported by experiments in which binding of 12-AS to vesicles was monitored by the fluorescence of the anthroyloxy group. When 12-AS was added to the vesicle suspension, there was an essentially instantaneous appearance of fluorescence of 12-AS followed by a slow rate of appearance of additional fluorescence (Figure 3A, lower trace). The ratio between the peak of instantaneous fluorescence and total fluorescence (measured for several minutes to about 1 h) depended inversely on the amount of 12-AS added to the vesicles (not shown). Since the anthroyloxy group fluoresces in lipid bilayers but not in water, this result can be explained by rapid entry of a small amount of 12-AS into vesicles. The slow phase for appearance of fluorescence reflects slow dissolution of 12-AS that precipitated from ethanol solution on addition to aqueous solution.

Flip-Flop of 12-AS Monitored by Adjustment of External pH. Because of the much lower solubility of 12-AS in water as compared with that of palmitate, it was not possible to prepare a soap solution of 12-AS to use in a stopped flow experiment as in Figure 1. Therefore, 12-AS was added to vesicles by cosonication to assure complete incorporation into the bilayer, and the transbilayer distribution of 12-AS was perturbed by changing the external pH as in Figure 1A. Figure 3B illustrates an experiment with vesicles containing trapped pyranin and 2 mol % cosonicated 12-AS. Two aliquots of KOH were added to these vesicles, each resulting in an instantaneous increase in pyranin fluorescence. As discussed above (Figure 1A), this increase in fluorescence reflects an increase in pH_{in} caused by fast redistribution of 12-AS to the external leaflet, in response to the increase in external pH to 7.87. Subsequently, two aliquots of oleic acid were added and the expected decreases in pH_{in} were observed, showing that the vesicles were still intact.

The pH jump experiment was repeated with the stopped flow method. A suspension of vesicles with 2 mol % cosonicated 12-AS and trapped pyranin was mixed with an equal volume of distilled water to establish the control level of fluorescence. Subsequently, the suspension of vesicles with 12-AS was mixed rapidly in the stopped flow apparatus with KOH to increase pH_{out} to 7.8. A small increase in fluorescence was found within the mixing time of the experiment (data not shown). This indicated that the 12-AS had already re-equilibrated between the two leaflets of the membrane within the 5−10 ms mixing time, which requires extremely fast flip-flop.

Transfer of 12-As between Populations of Vesicles. As mentioned, it has been proposed that anthroyloxy-labeled FA flip-flop slowly across bilayers (Storch & Kleinfeld, 1986; Kleinfeld & Storch, 1993) because the rate of transfer of these FA between populations of vesicles fits a double exponential function (Kleinfeld & Storch, 1993). This result was interpreted to mean that a relatively fast component of the transfer process corresponds to transfer of FA from the

outer leaflet of a donor to an acceptor population of vesicles (determined by K_{off}) and that a relatively slow component corresponds to the transfer of FA initially on the inner leaflets of the donor bilayers (determined by k_{flip} and k_{off}). We illustrate this in Scheme 4:

donor vesicles water acceptor vesicles

$$FA_{in} \underset{k_{flip}}{\overset{k_{flip}}{\rightleftharpoons}} FA_{out} \underset{k_{on}}{\overset{k_{off}}{\rightleftharpoons}} FA_{water} \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} FA_{out} \underset{k_{flip}}{\overset{k_{flip}}{\rightleftharpoons}} FA_{in} \quad (4)$$

Here k_{flip} and k_{off} represent the (pseudo-) unimolecular rate constants for flip-flop and dissociation (solvation) of the FA, respectively. k_{on} is the extremely fast binding rate constant.

Since our results (Figure 3) suggest that flip-flop of 12-AS is very fast, we re-examined the transfer assay. To eliminate potential problems with solubilization of 12-AS, we added 12-AS to vesicles by cosonication. In one protocol, we used the pyranin assay, and in a second, we monitored quenching of the fluorescence of 12-AS by NBD-labeled phospholipids (Kleinfeld & Storch, 1993).

Transfer Assay Reflected by Changes in the Fluorescence of Pyranin. Donor vesicles in these experiments contained 2 mol % cosonicated 12-AS as well as trapped pyranin. When acceptor vesicles (with no pyranin) were added, there was a slow increase in the fluorescence of pyranin, which leveled off according to a single exponential (Figure 4A). The rate constant for this process was $k_{obs} = 0.0076\ s^{-1}$. This increase in the fluorescence of pyranin reflects the transfer of 12-AS from the inner to the outer leaflet of the donor vesicles and transfer to the acceptor vesicles. In a separate experiment (Figure 4B), pyranin was present only in acceptor vesicles and the transfer of 12-AS from donor vesicles followed by flip-flop in the acceptor vesicles was monitored by a decrease in the fluorescence of pyranin. The rate for transfer in this experiment was a single exponential with a value of $k_{obs} = 0.0077\ s^{-1}$, very close to the rate observed in the first experiment. This means that only one event determines the overall rate in the transfer process and that all other events must be much faster. We cannot distinguish from these experiments, however, whether flip-flop or dissociation of FA from the donor vesicles was the rate-controlling event that was measured.

Transfer Assay Using NBD Quenching of 12-AS. The fluorescence of 12-AS in PC vesicles was first monitored to determine the extent of photobleaching. A significant decrease in fluorescence was seen over a time course of 1000 s (Figure 4C, upper trace). The lower trace in Figure 4C shows fluorescence changes of 12-AS associated with transfer from donor vesicles (containing 2 mol % cosonicated 12-AS) to acceptor vesicles with 10 mol % cosonicated NBD-PE. The observed decrease in fluorescence of 12-AS is due to transfer of some of the 12-AS from the donor to the acceptor vesicles, where the fluorescence is quenched by NBD, as well as photobleaching of 12-AS. This curve was better fit to a double exponential than a single exponential. The double exponential fit yielded a fast rate constant of $k_f = 0.0078\ s^{-1}$ and a slow rate constant of $k_s = 0.0017\ s^{-1}$. There is a close correspondence between the rate constant for transfer of FA measured by the pyranin assay and the fast component found in the NBD assay. We thus attribute the fast component to 12-AS transfer and infer

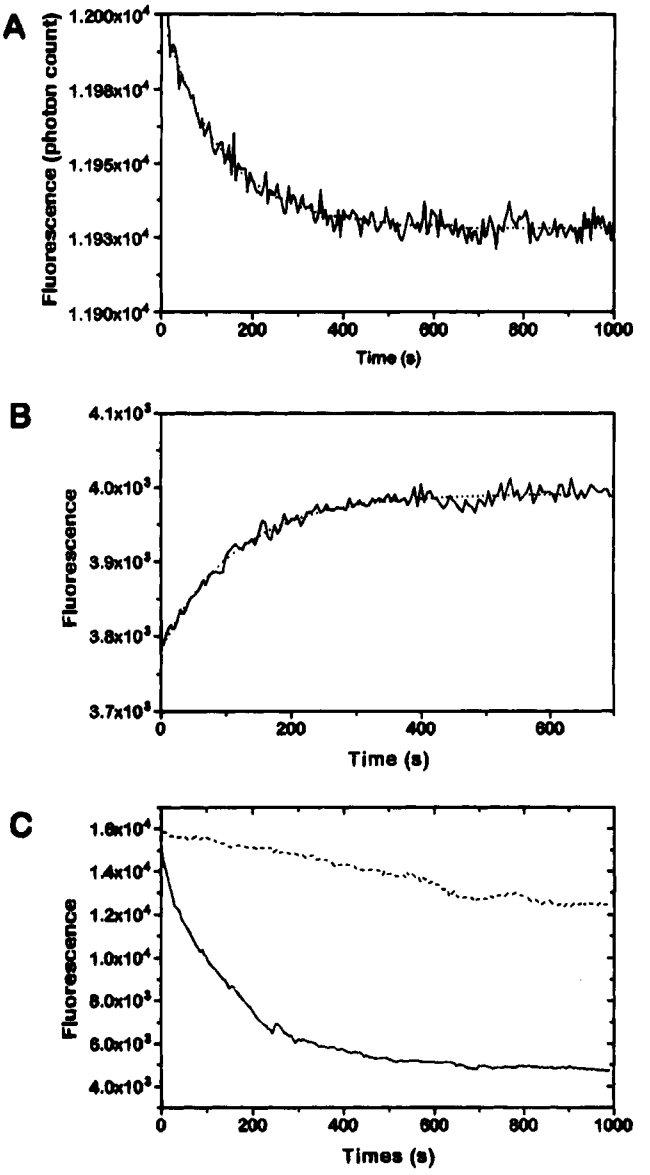

FIGURE 4: 12-AS transfer assays. (A) Pyranin fluorescence upon mixing of donor vesicles (110 μM PC) containing 2 mol % cosonicated 12-AS and trapped pyranin with acceptor vesicles (475 μM PC). (B) Pyranin fluorescence upon mixing of donor vesicles (195 μM PC) containing 2 mol % 12-AS with acceptor vesicles (240 μM PC) containing trapped pyranin. The dotted lines represent a single exponential fit of the data. (C) 12-AS transfer assay using NBD quenching. Fluorescence was sampled in 5 s intervals. The dashed line shows 12-AS fluorescence of donor vesicles (13 μM PC) containing 2 mol % cosonicated 12-AS. The decrease in fluorescence reflects a significant rate of photobleaching. The solid line shows a transfer assay in which donor vesicles were mixed with acceptor vesicles (170 μM PC with 10 mol % NBD). The decrease of the fluorescence reflects both quenching and photobleaching.

that the slow component seen in the NBD assay is an artifact not related to FA transfer.[2]

It can now be concluded that only the dissociation of 12-AS from the donor vesicles determines the rate of transfer. If flip-flop were slow and dissociation very fast, an instantaneous decrease in 12-AS fluorescence upon mixing of donor and acceptor vesicles would have been observed in

[2] It was not possible to account for the entire slow component by photobleaching, on the basis of our control experiment shown. Kleinfeld and Storch (1986) reported that they performed experiments under conditions where no photobleaching occurred.

the NBD assay because of rapid transfer of 12-AS from the outer leaflet. This would be followed by a slow decay representing the flip-flip of 12-AS from the inner leaflet of the donor vesicles. However, this was not observed. The results of the transfer experiments permit calculation of the dissociation rate constant (k_{off}) from the observed rate constant (k_{obs}) of the single exponential changes in fluorescence as follows. Since k_{flip} is extremely fast, scheme 4 can be reduced to

$$FA_D \underset{k_{on}}{\overset{k_{off}}{\rightleftharpoons}} FA_{water} \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} FA_A \quad (5)$$

where FA_D denotes the FA on the donor vesicles, FA_{water} the FA in the water, and FA_A the FA on the acceptor vesicles. For simplicity, we assume k_{off} and k_{on} are the same for donor and acceptor vesicles. Upon mixing of the donor and acceptor vesicles, the following differential equations apply for the moles of FA in the donor vesicles (n_D) and the moles of FA in the water (n_w).

$$\frac{d(n_D)}{dt} = -k_{off}n_D + k_{on}n_W D \quad (6)$$

$$\frac{d(n_W)}{dt} = k_{off}n_D - k_{on}n_W D + k_{off}n_A - k_{on}n_W A \quad (7)$$

n_A denotes the amount of moles of FA on the acceptor vesicles, D the concentration of donor vesicles (moles of PC), and A the concentration of acceptor vesicles (moles of PC). Assuming that the concentration of FA in the water phase is very low, $d(n_w)/dt = 0$ at all times and eq 6 can be solved:

$$n_D(t) = T\left(1 - \frac{D}{D+A}\right)e^{-k_{off}t} + T\frac{D}{D+A} \quad (8)$$

where T denotes the total amount of FA present in the system. It follows that n_D decreases exponentially from an initial (T) to final ($TD/(D + A)$) value. The final value depends on the ratio of donor to acceptor vesicles, but the observed rate constant does not because the binding to the acceptor vesicles does not saturate, as it could in the case of BSA (Daniels et al., 1985). In the pyranin transfer assays, the change in pyranin fluorescence is proportional to the amount of FA that has been transferred. Therefore, $k_{obs} = k_{off} = 0.0077$ s^{-1}.

DISCUSSION

A direct attempt to measure the rate of flip-flop of FA in SUV by stopped flow experiments showed that FA added to the outside of small unilamellar vesicles equilibrated (≥80%) between the outer and inner leaflets of bilayers within the dead time of the instrument (5−10 ms). The small decay representing the tail of the exponential in SUV experiments suggests that the flip-flop rate is in the low millisecond and not the microsecond time range. This demonstrates that the rate of transbilayer flip-flop of unionized FA in SUV is minimally 100 times faster than previously reported (Kamp & Hamilton, 1992, 1993). NMR spectra of FA in SUVs do not show separate signals for FA in each leaflet, consistent with an exchange rate of milliseconds or faster (Hamilton, 1995).

In interpreting the results of rate measurements with LUV, it is important to consider artifacts that might arise from sample inhomogeneities and the mixing procedures. We carried out detailed characterizations of LUV, to assess their structure and homogeneity. These characterizations showed an absence of SUV (external to the LUV), which could make the rate appear faster, and of multilamellar vesicles or much larger LUV, which could make the rate appear slower than that observed for the LUV samples. The presence of small vesicles trapped inside LUV as observed for a fraction of the LUV in one sample preparation would not affect our results significantly because they comprise <5% of total PC. The fluorescence changes with LUV also cannot be due to mixing artifacts or precipitation of FA since the same mixing conditions were used for SUV (which showed a faster decay) and the rates for different FA were similar for LUV despite their much different aqueous solubilities. Thus, we conclude that the results for LUV reflect slower flip-flop of FA than in SUV. Flip-flop of FA (lauric, myristic, palmitic, oleic, and stearic) in LUV of 100 nm in diameter was fast but measurable ($k_{FAH} \sim 15$ s^{-1}) and did not depend on FA chain length. From our studies, it is not possible to make an accurate quantitative comparison between SUV and LUV, but the rate of FA flip-flop must be >3-fold slower in LUV. Whether the lower curvature of biological membranes compared to LUV has an additional effect on the kinetics is not known. Nevertheless, the rate of spontaneous transbilayer movement in cell membranes could be 2 orders of magnitude slower than in PC LUV and still be faster than intracellular utilization of FA (Noy et al., 1986a).

If we assume that flip-flop is diffusion through a free volume in the membrane (Träuble, 1971), the theoretical upper time limit to the rate is the time to achieve separation of two neighboring head groups by approximately one chain diameter. Pace and Chan (1982) calculated this to be 10^{-8} s. If the space required were larger, for instance the separation of two neighboring phospholipids by one phospholipid head group, as suggested for transmembrane movement of H_2O (Haines, 1994), the theoretical limit would be 10^{-6} s. Such a separation would be required for the flip-flop of diacylglycerols, which exhibit flip-flop in PC vesicles with $t_{1/2} = 11$ ms at 38 °C (Hamilton et al., 1991). Events that might slow the actual rate of flip-flop of FA are the breaking of hydrogen bonds linked to the COOH group, conformational changes accompanying the requisite reorientation in the bilayer, and transverse diffusion of the molecule through the core of the membrane, although the latter is very fast for small molecules (Haines, 1994; Träuble, 1971; Lieb & Stein, 1969).

Another contributor to the rate would be the total free volume of the membranes, which decreases as the curvature decreases. This effect could account for the dependence of the rate of flip-flop on the curvature of vesicles. The independence of the rate of flip-flop on the chain length of FA also can be understood in this context. Thus, the energy of activation for flip-flop must be the energy needed to create a void volume that extends across the leaflet to which FA diffuses. This energy is a property of the bilayer and should be independent of the chain length of FA. Supporting evidence for this idea is that the rate of lateral diffusion in bilayers is independent of the extent to which amphipaths extend into the apolar region of bilayers (Balcom & Peterson, 1993; Vaz et al., 1985). In contrast to the rate of flip-flop, the rate of desorption of FA from a phospholipid interface depends strongly on the FA chain length and decreases by a factor of 10 for addition of two methylene

units (Daniels et al., 1985; Zhang & Hamilton, 1995) (if this were true for flip-flop of FA, there would be a 1000-fold difference in the rates for lauric acid and stearic acid). These findings are analogous to those for phospholipids; in a phospholipid bilayer, the rate of flip-flop decreases by a factor of <2 for addition of two methylene units in one chain, whereas the desorption rate decreases by a factor of 8 (Homan & Pownall, 1988).

Our observation of the extremely fast unfacilitated diffusion (flip-flop) of FA across phospholipid bilayers removes an important corollary of the protein-mediated transport hypothesis, that FA transbilayer movement in the absence of proteins is slow (Storch, 1990), and challenges the view that proteins are necessary for transport of FA across biomembranes. Specifically, the present evidence argues against models in which a protein acts to translocate FA through the lipid bilayer (Higgins, 1994). Another premise for a transport protein is that FA exist only as anions in the membrane and that FA are transported in the anionic form (Stremmel et al., 1985; Potter et al., 1989). However, NMR measurements show that FA have an apparent pK_a of ~7.5 in a phospholipid interface, meaning that there is an equal population of un-ionized FA (Cistola & Hamilton, 1986; Hamilton, 1995). The pyranin assay directly (Kamp & Hamilton, 1992) verifies the NMR prediction of a significant pool of un-ionized FA in the membrane.

Measurements of the transfer of natural (Daniels et al., 1984) and pyrene-labeled FA (Doody et al., 1980) between phospholipid vesicles have suggested that the diffusion through the bilayer is faster than desorption of FA from the vesicle interface. Since the only potentially contradictory biophysical data are for anthroyloxy FA (Kleinfeld & Storch, 1993), we re-examined flip-flop of anthroyloxy FA in vesicles (Kamp & Hamilton, 1993) and the transfer of these acids between vesicle populations (Kleinfeld & Storch, 1993). We showed in this regard that our previous conclusion that 12-AS flip-flop is slow is incorrect because of an artifact caused by precipitation of 12-AS added to the vesicle suspension. When 12-AS was cosonicated with PC to ensure solubilization in the bilayer, a rapid response of pyranin fluorescence was obtained by increasing the external pH, which directly demonstrates rapid flip-flop of 12-AS in vesicles (Figure 3B).

Additionally, we showed that the kinetics of transfer of 12-AS between vesicles, measured by the pyranin assay, were described by a single exponential (Figure 4A,B). Thus, either flip-flop or transfer was extremely fast. Our protocol used vesicles prepared by sonication to incorporate 12-AS in both leaflets of the bilayer and to ensure that 12-AS did not precipitate in an unbound form. The rate of quenching of 12-AS representing the transfer of 12-AS from cosonicated vesicles to vesicles with NBD showed only a slow component (Figure 4C), indicating that flip-flop must be fast relative to desorption, consistent with the findings of the first group of experiments.

The k_{off} for 12-AS (0.0077 s^{-1}) from phospholipid vesicles is much lower (~100-fold) than that found for stearic acid (Daniels et al., 1985) and is comparable to that for a 22-carbon saturated FA (Zhang & Hamilton, 1995). Assuming that k_{on} is defined by the similar diffusion limit for both 12-AS and naturally occurring FA, the equilibrium constant between bilayer and water ($K_{eq} = k_{on}/k_{off}$) for 12-AS must be more in favor of the bilayer compared to unlabeled FA. This means that 12-AS is much more hydrophobic than naturally occurring FA, consistent with our findings that a large fraction of 12-AS, dissolved in ethanol, precipitates prior to binding when added to vesicles suspended in water (Figure 3A), as compared to palmitate and oleate.

The independence of the rate of flip-flop for natural FA on the chain length and the rapid rate for the flip-flop of bulky FA like anthroyloxy FA, as shown above, and pyrene-labeled FA (Kamp & Hamilton, 1993) likely has physiologic significance for other molecules. For example, mediators of inflammation that are the products of cycloxygenase-catalyzed oxidation of polyunsaturated FA are also bulky weakly polar molecules. The data presented above suggest that these kinds of molecules will move rapidly and spontaneously across bilayers and that their movement between cells or compartments in cells will not be limited by the rate of spontaneous transbilayer movement.

ACKNOWLEDGMENT

We thank Dr. Kantrowitz of Boston College for use of the stopped flow equipment and Christine Soltys for assistance in these measurements. We also thank Don Gantz for EM measurements and Dr. Tony Scotto for assistance in the preparation of LUV.

REFERENCES

Abumrad, N. A., Perkins, R. C., Park, J. H., & Park, C. R. (1981) *J. Biol. Chem. 256*, 9183−9191.
Abumrad, N. A., El-Maghrabi, M. R., Amri, E.-Z., Lopez, E., & Grimaldi, P. A. (1993) *J. Biol. Chem. 268*, 17665−17668.
Anel, A., Richieri, G. V., & Kleinfeld, A. M. (1993) *Biochemistry 32*, 530−536.
Balcom, B. J., & Peterson, N. O. (1993) *Biophys. J. 65*, 630−637.
Bartlett, G. R. (1959) *J. Biol. Chem. 234*, 466−468.
Cooper, R., Noy, N., & Zakim, D. (1987) *Biochemistry 26*, 5890−5896.
Daniels, C., Noy, N., & Zakim, D. (1985) *Biochemistry 24*, 3286−3292.
Doody, M. C., Pownall, H. J., Kao, Y. J., & Smith, L. C. (1980) *Biochemistry 19*, 108−116.
Dreyer, C., Krey, G., Keller, H., Givel, F., Helftenbein, G., & Wahli, W. (1992) *Cell 68*, 879−887.
Haines, T. (1994) *FEBS Lett. 364*, 115−120.
Hamilton, J. A. (1995) in *Carbon-13 NMR Spectroscopy of Biological Systems*, pp 117−157, Academic Press, San Diego.
Hamilton, J. A., Civelek, V. N., Kamp, F., Tornheim, K., & Corkey, B. E. (1994) *J. Biol. Chem. 269*, 20852−20856.
Higgins, C. F. (1994) *Cell 79*, 393−395.
Homan, R., & Pownall, H. (1987) *J. Am. Chem. Soc. 109*, 4759−4760.
Homan, R., & Pownall, H. (1988) *Biochim. Biophys. Acta 938*, 155−166.
Hope, M. J., & Cullis, P. R. (1987) *J. Biol. Chem. 2022*, 4360−4366.
Hope, M. J., Bally, M. B., Webb, G., & Cullis, P. R. (1985) *Biochim. Biophys. Acta 812*, 55−65.
Kamp, F., & Hamilton, J. A. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 11367−11370.
Kamp, F., & Hamilton, J. A. (1993) *Biochemistry 32*, 11074−11087.
Kleinfeld, A. M., & Storch, J. (1993) *Biochemistry 32*, 2053−2061.
Lieb, W. R., & Stein, W. D. (1969) *Nature 224*, 240−243.
McPheat, W. L., & Green, S. (1992) *EMBO J. 11*, 433−439.
Neely, J. R., & Morgan, H. E. (1974) *Annu. Rev. Physiol. 36*, 413−459.
Noy, N., Donnelly, T. M., & Zakim, D. (1986a) *Biochemistry 25*, 2013−2021.
Noy, N., Donnelly, T. M., Cooper, R. B., & Zakim, D. (1986b) *Biochim. Biophys. Acta 1003*, 125−130.
Olsen, F., Hunt, C. A., Szoka, F. C., Vail, W. J., & Papahadjopoulos, D. (1979) *Biochim. Biophys. Acta 557*, 9−23.

Ordway, R. W., Walsh, J. V., Jr., & Singer, J. J. (1989) *Science 244*, 1176−1179.
Pace, R. J., & Chan, S. I. (1982) *J. Chem. Phys. 76*, 4241−4247.
Potter, B. J., Sorrentino, D., & Berk, P. D. (1989) *Annu. Rev. Nutr. 9*, 253−270.
Schwieterman, W., Sorrentino, D., Potter, B. J., Rand, J., Kiang, C.-L., Stump, D., & Berk, P. D. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 359−363.
Smith, I. C. P., & Ekiel, I. H. (1984) in *Phosphorus NMR: Principles and Applications* (Gorenstein, D., Ed.) pp 447−475, Academic Press, Orlando.
Spector, A. A., Steinberg, D., & Tanaka, A. (1965) *J. Biol. Chem. 240*, 1032−1041.
Storch, J., & Kleinfeld, A. M. (1986) *Biochemistry 25*, 1717−1726.
Storch, J., Lechene, C., & Kleinfeld, A. M. (1991) *J. Biol. Chem. 266*, 13473−13476.
Stremmel, W., Strohmeyer, G., Borchard, F., Kochwa, S., & Berk, P. D. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 4−8.
Träuble, H. (1971) *J. Membr. Biol. 4*, 193−208.
Tugwood, J. D., Issemann, I., Anderson, R. G., Bundell, K. R., McPheat, W. L., & Green, S. (1992) *EMBO J. 11*, 433−439.
Vaz, W. L., Clegg, R. M., & Hallman, D. (1985) *Biochemistry 24*, 781−786.
Wilschut, J., Scholma, J., Eastman, S. J., Hope, M. J., & Cullis, P. R. (1992) *Biochemistry 31*, 2629−2636.
Wrigglesworth, J. M., Sharpe, M. A., & Cooper, C. E. (1993) *Biochem. Soc. Trans. 21*, 781−784.
Zhang, B., Marcus, S. L., Miyata, K. S., Subramani, S., Capone, J. P., & Rachubinski, R. A. (1993) *J. Biol. Chem. 268*, 12939−12945.
Zhang, F., & Hamilton, J. A. (1995) *Biophys. J. 68*, A432.

BI950375A