Movement of Fatty Acids, Fatty Acid Analogues, and Bile Acids across Phospholipid Bilayers†

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Appendix: Kinetics of Fatty Acid-Mediated Proton Movement across Small Unilamellar Vesicles

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ABSTRACT: How lipophilic acids move across membranes, either model or biological, is the subject of controversy. We describe experiments which better define the mechanism and rates in protein-free phospholipid bilayers. The transbilayer movement of lipophilic acids [fatty acids (FA), covalently-labeled FA, bile acids, and retinoic acid] was monitored by entrapping pyranin, a water-soluble, pH-sensitive fluorescent molecule to measure pH inside unilamellar vesicles [Kamp, F., & Hamilton, J. A. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 11367–11370]. Equations for the pseudo-unimolecular rate constants for transbilayer movement of un-ionized (k_{FAH}) and ionized (k_{FA^-}) acids are derived. All FA studied (octanoic, lauric, myristic, palmitic, stearic, oleic, elaidic, linoleic, linolelaidic, and arachidonic) and retinoic acid exhibited rapid transbilayer movement ($t_{1/2} < 1$ s) via the un-ionized form across small unilamellar egg phosphatidylcholine (PC) vesicles. FA produced by phospholipase A_2 in the outer leaflet of PC vesicles equilibrated rapidly to the inner leaflet. Ionized FA showed enhanced transbilayer movement ($k_{FA^-} = 0.029$ s^{-1}) in the presence of equimolar valinomycin. The three FA analogues [12-(9-anthroyloxy)stearic acid, 5-doxylstearic acid, and 1-pyrenenonanoic acid] moved across PC bilayers via the un-ionized form; except for the anthroyloxy FA ($k_{FAH} = 4.8 \times 10^{-3}$ s^{-1}), the rates were too fast to measure ($t_{1/2} < 1$ s). The rate for cholic acid (CA) transbilayer movement was slow ($k_{CAH} = 0.056$ s^{-1}) compared to that of the more hydrophobic bile acids, deoxy- and chenodeoxycholic acid ($t_{1/2} < 1$ s). The taurine conjugates of the three bile acids did not cross the bilayer ($t_{1/2} > 1$ h). A further application of the pyranin method was to measure the partitioning of FA and bile acids among water, albumin, and PC vesicles. Our results show that the ability of lipophilic acids to permeate a PC bilayer rapidly is dependent on the presence of the un-ionized acid in the membrane interface. Considering the fast unfacilitated movement of FA across protein-free phospholipid bilayers, it is unlikely that there is a universal need for a transport protein to enhance movement of FA across membrane bilayers. Physiological implications of proton movement accompanying fast movement of un-ionized lipophilic acids (and the consequent generation of a pH gradient) are discussed.

Unesterified fatty acids (FA)[1] constitute a minor fraction of lipids in the body but play central roles in metabolism and in regulating cellular processes. FA are delivered to cells, primarily by plasma albumin (Spector, 1986) or by hydrolysis of plasma triglycerides, for resynthesis of esterified lipids or for oxidative energy (Neely & Morgan, 1974). When released from intracellular storage sites, FA are either utilized or move out of cells to the plasma compartment for delivery to tissues requiring FA. Additionally, FA participate in signal transduction pathways (Hannigan & Williams, 1991), activate K^+ channel activity (Ordway et al., 1989) and Na^+/Ca^{2+} exchange (Philipson & Ward, 1985), modulate uptake of plasma lipoproteins (Bihain et al., 1989), and uncouple oxidative phosphorylation (Skulachev, 1991). In myocardial ischemia FA reportedly cause diverse electrophysiological and other perturbations of cell functions (Corr et al., 1984).

The question of whether FA can freely move across a phospholipid membrane or whether proteins are essential for their transport has been one of long-standing interest (Spector, 1986; Storch, 1990; Paulussen & Veerkamp, 1990; Veerkamp et al., 1991; Kamp & Hamilton, 1992). An answer is difficult to achieve: on the cellular level, the processes of transport are complex and difficult to separate into individual mechanistic components. On the model system level (as well as the cellular level) the simple structure of the FA has been considered an obstacle in monitoring the movement of the molecule. Therefore, fluorescent probes have been attached to FA to monitor transbilayer movement in model membranes and in cells. Studies of pyrene-labeled FA (Doody et al., 1980) and FA labeled with an anthroyloxyl group (Storch & Kleinfeld, 1986; Kleinfeld & Storch, 1993) led to different

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[1] Abbreviations used: BSA, bovine serum albumin; CA, cholic acid; CDCA, chenodeoxycholic acid; DCA, deoxycholic acid; FA, fatty acid; OA, oleic acid; PA, palmitic acid; PC, phosphatidylcholine; PLA_2, phospholipase, A_2; SUV, small unilamellar vesicle(s); k_b, relaxation rate constant of "background" proton leak; k_t, relaxation rate constant of FA-mediated plus background proton leak; k_{FA^-}, pseudo-unimolecular rate constant of flip-flop of ionized FA; k_{FAH}, pseudo-unimolecular rate constant of flip-flop of un-ionized FA.

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predictions of the rate of transbilayer movement of FA across phospholipid bilayers. Transbilayer movement was not evaluated directly but by monitoring transfer of the FA probe between phospholipid vesicles. Additionally, the rate of transbilayer movement of fluorescent-labeled FA may depend on the nature of the attached fluorophore and differ from that of native FA. Studies with cell systems and isolated biological membranes have been interpreted to suggest passive diffusion (Bröring et al., 1989; Cooper et al., 1989), protein-mediated transport (Abumrad et al., 1981; Schwieterman et al., 1988; Diede et al., 1992), or both mechanisms (Storch et al., 1991; Veerkamp et al., 1991).

Recently we reported a new technique to monitor unfacilitated transbilayer movement (flip-flop) of native FA across phospholipid bilayers (Kamp & Hamilton, 1992). This fluorescence-based approach differs from those mentioned above in that the fluorescent probe is not attached to the FA. Furthermore, the probe does not interact directly with FA or the phospholipid matrix. Long-chain FA were added to egg phosphatidylcholine (PC) vesicles (with and without cholesterol) containing entrapped pyranin, a water-soluble fluorescent pH indicator. The pH inside the vesicles (pH_{in}) decreased instantaneously ($t_{1/2} < 2$ s), demonstrating fast movement of un-ionized FA to the inner leaflet of the vesicle, followed by ionization of about half of these FA. The effect on pH_{in} could be immediately and fully reversed by extraction of the FA with albumin. These findings are in apparent conflict with studies of anthroyloxyl-labeled FA, suggesting a slow overall rate of transbilayer movement and a faster movement of the ionized form than the un-ionized form (Storch & Kleinfeld, 1986; Kleinfeld & Storch, 1993).

In this study we examine the transbilayer movement of several native FA and other lipophilic acids in small unilamellar vesicles (SUV's). FA are introduced into the outer leaflet of SUV's by addition of solubilized FA (K^+ soap or ethanolic solution) to the SUV suspension or by the action of phospholipase A_2 and the response of pyranin fluorescence measured. The rate of transbilayer movement of ionized FA is also measured in the presence of valinomycin. We derive equations for the rate of transbilayer movement of un-ionized and ionized lipophilic acids across a phospholipid bilayer to evaluate the pseudo-unimolecular rate constants for flip-flop of the uncharged and charged forms (see Appendix). The pyranin assay is used to compare the transbilayer movement of native FA with nitroxide- and fluorescent-labeled FA. Finally, our methodology is applied to make quantitative measurements of partitioning of FA and bile acids between SUV's and albumin.

MATERIALS AND METHODS

SUV's with entrapped pyranin were made by sonication. Egg phosphatidylcholine (PC) (Avanti Polar Lipids, Pelham, AL) (45 mg) in chloroform was pipetted into a scintillation vial. For vesicles with cosonicated oleic acid, the required amount of oleic acid, dissolved in chloroform, was first mixed with PC. Chloroform was evaporated under a stream of N_2 gas and the lipid film dried under vacuum for 1 h. Subsequently, 1.8 mL of Hepes/KOH buffer (100 mM, pH 7.40) with 0.5 mM pyranin (Kodak) was added and the film vortexed for 1 min, hydrated at 4 °C overnight, and vortexed again for 1 min. The lipid suspension was sonicated on a Branson sonifier with a microtip for 1 h at medium power in a pulsed mode (30% duty cycle) on ice and under a gentle stream of N_2 gas. The obtained clarified SUV suspension was centrifuged (20 min) with a table centrifuge to remove metal particles. To remove untrapped pyranin, the outer volume of the SUV suspension was replaced by 100 mM Hepes/KOH (pH 7.40) using gel filtration (Kamp & Hamilton, 1992). The final PC concentration of the SUV suspension was measured by a phosphorous assay (Bartlett, 1959).

For electron microscopy, samples were prepared by dilution of hydrated vesicle samples with buffer to 0.5–1.0 mg/mL lipid. Samples were applied to 400-mesh copper grids covered with carbon-coated Formvar and stained with 1% sodium phosphotungstate solution at pH 7.5. After drying in air, samples were examined with a Philips CM-12 electron microscope at 100 kV.

Fluorescence measurements were carried out with a Perkin-Elmer fluorimeter with a stirred cuvette at room temperature. In all experiments an aliquot of the suspension of PC vesicles with trapped pyranin was added to 2.0 mL of Hepes/KOH buffer (pH 7.40). External pH (pH_{out}) was measured with a pH minielectrode (Microelectrodes, Londonderry, NH), and internal pH (pH_{in}) by the pyranin fluorescence (excitation wavelength, 455 nm; emission wavelength, 509 nm). The relation between pH_{in} and pyranin fluorescence was calibrated as described (Kamp & Hamilton, 1992). The response time of the pH_{in} measurement (~2 s) was limited by the response time of the fluorimeter and mixing in the cuvette.

Stock solutions of 10 mM native FA (Avanti Polar Lipids, Pelham, AL) were made in H_2O at pH > 11 (except for stearic acid) or in ethanol. We previously showed that equivalent results were obtained for long-chain FA (added to vesicles in ethanol solution or as the K^+ soap (Kamp & Hamilton, 1992). Aqueous solutions of 1–4 mM FA-free bovine serum albumin (BSA; fraction V, Sigma) were made as before (Hamilton & Cistola, 1986). Stock solutions of retinoic acid (Sigma), 1-pyrenenonanoic acid, 5-doxylstearic acid, and 12-(9-anthroyloxy)stearic acid (Molecular Probes, Eugene, OR) were made in ethanol. 1-Pyrenenonanoic acid, 5-doxylstearic acid, and 12-(9-anthroyloxy)stearic acid displayed no measurable fluorescence at the excitation and emission wavelengths used for pyranin. Stock solutions (100 mM) of bile salts (Calbiochem) were made in distilled water. When BSA was loaded with FA or bile acids, the required amount of an aqueous stock solution of FA or bile acid was added to aqueous BSA and mixed by gently shaking prior to addition to the vesicles. Stock solutions of 1 mg/mL nigericin (Sigma) and valinomycin (Sigma) were prepared in ethanol. A stock solution (0.01 unit/μL; 1 unit of phospholipase A_2 releases 1 μmol of FA/min, from phospholipids) of phospholipase A_2 (Sigma) was prepared in 100 mM Hepes/KOH (pH 7.4) containing 5 mM $CaCl_2$.

RESULTS

Proton Leak. Vesicles were first tested for proton leakage in the absence and presence of ionophores. In the experiment of Figure 1A, KOH was added to vesicles with trapped pyranin dispersed in Hepes/KOH buffer (initial $pH_{in} = pH_{out} = 7.4$) to increase pH_{out} to 7.6. pH_{in} increased very slowly ($t_{1/2} \simeq$ hours), as indicated by the slow rise in pyranin fluorescence. This result was expected because proton leakage under these circumstances is limited by the buildup of a diffusion potential, which can only be abolished by extremely slow counterflow of K^+ (Deamer & Nichols, 1989), as illustrated schematically by Figure 1B. Addition of valinomycin enhanced counterflow of K^+ (Figure 1C) and increased the proton flux, as shown by the increasing pyranin fluorescence (Figure 1A). Upon addition of nigericin, which rapidly exchanges K^+ for H^+, the pH gradient collapsed instantaneously (Figure 1A).

Rapid Transbilayer Movement of Un-ionized FA. To monitor movement of FA across the bilayer of well-sealed

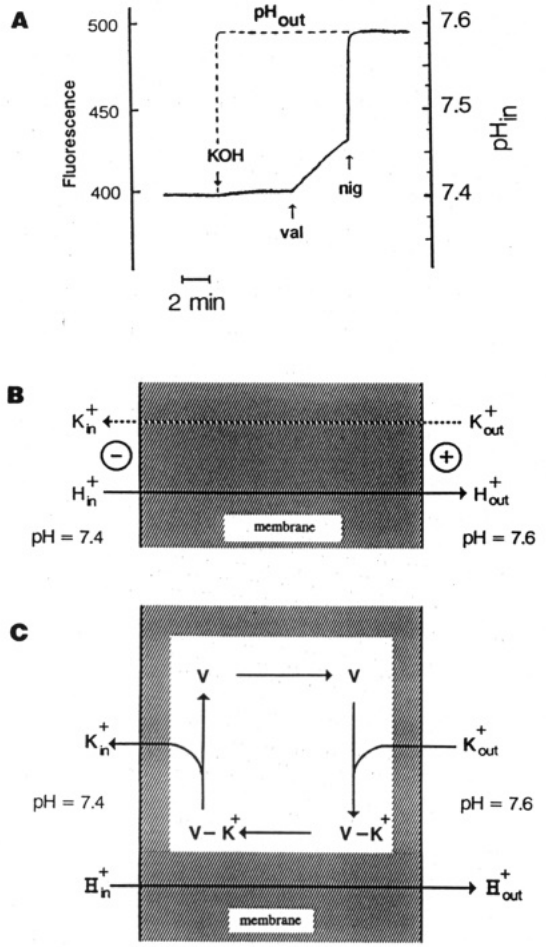


FIGURE 1: Proton leak in small unilamellar vesicles: vesicles containing pyranin in 2 mL of buffer (100 mM Hepes/KOH, pH 7.40). The PC concentration was 0.56 mg/mL (0.72 mM). (A) Generation and collapse of a pH gradient by adding KOH, 1 μg of valinomycin (val), and 1 μg of nigericin (nig). The dotted line represents the response of the pH electrode (pH$_{out}$). (B) An illustration of a membrane with a pH gradient of 0.2 unit. The K^+ concentration on both sides of the membrane is ~100 mM. Because of the low electrical capacitance of phospholipid bilayers, transfer of only a few protons generates a significant diffusion potential. Further proton leakage must be compensated by counterflux of co-ions (here K^+ ions). The latter limits the proton flow. (C) A membrane containing valinomycin, with a pH gradient across it [as in (B)]. Because of fast cyclic transfer of K^+ ions by valinomycin (V), the background proton flux is released.

vesicles, solubilized FA (K^+ soap or ethanolic solution) was added to the external buffer. Figure 2A shows an experiment in which an aliquot of oleic acid (2.2 mol % with respect to PC) was added to vesicles (initial pH$_{in}$ = pH$_{out}$ = 7.40). The fluorescence of the trapped pyranin decreased instantaneously (within 2 s) to a value corresponding to pH$_{in}$ 7.13. The generated pH gradient was maintained over hours (not shown), demonstrating that the proton leak was not different from that observed without added FA (Figure 1A). Higher amounts (up to 14 mol %) of oleic acid gave larger pH$_{in}$ decreases, but no effects on proton leak were found (not shown).

The preceding protocol (Figure 2A) entailed preparation of vesicles without added FA (which generally contain <0.2 mol % endogenous FA; Kamp & Hamilton, 1992), and monitored movement of FA from the outer to inner leaflet ("flip"). To monitor movement of FA in the opposite direction ("flop"), we prepared vesicles with 2 mol % oleic acid (by cosonication of both lipids in buffer) and then added BSA to the external buffer to extract FA from these vesicles (Figure 2B). The fluorescence showed an instantaneous increase upon addition of BSA, corresponding to an increase in pH$_{in}$ to 7.76.

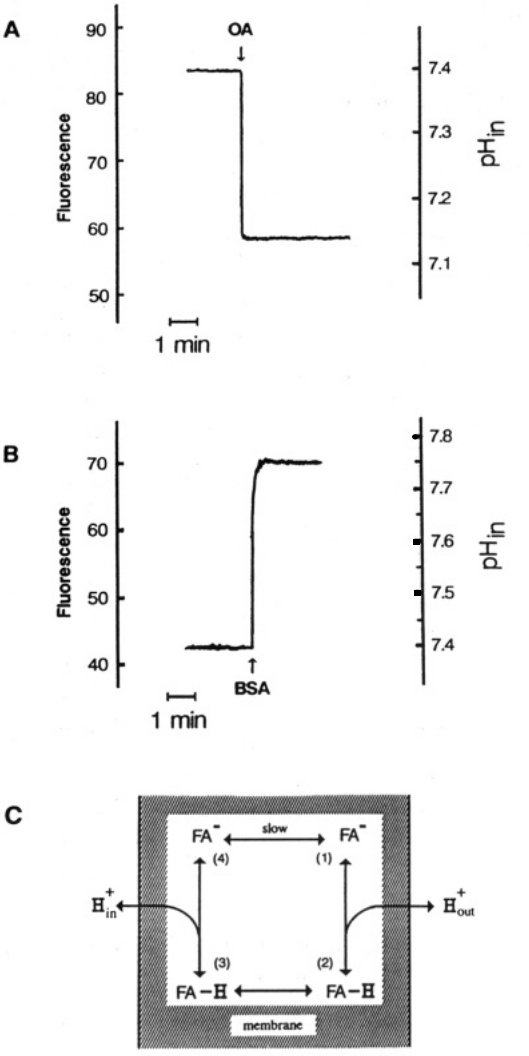


FIGURE 2: Rapid transbilayer movement of un-ionized FA. (A) To vesicles (1340 nmol of PC) in 2 mL of buffer was added 30 nmol of aqueous potassium oleate (OA). The internal pH dropped instantaneously, as shown by the pyranin fluorescence. (B) To vesicles (250 nmol of PC) with 2 mol % cosonicated oleic acid (5 nmol) in 2 mL of buffer was added an aliquot of 12 nmol of FA-free BSA, causing an instantaneous increase in internal pH. (C) Schematic representation of transbilayer movement of fatty acids (FA) across a membrane. FA can be in four states: the head group ionized or un-ionized (protonated), at either the inner or outer surface. The horizontal transitions represent the flip-flop reactions. All transitions are reversible. We hypothesize that all reactions are fast, except for the flip-flop of the ionized FA.

In a related experiment oleic acid was added to vesicles and allowed to equilibrate between both leaflets (as in Figure 2A); BSA was then added and instantaneously reversed the pH drop caused by addition of oleic acid (Kamp & Hamilton, 1992; see also Figure 5A below).

The above results can be explained (Kamp & Hamilton, 1992) by fast transbilayer movement of un-ionized FA compared to ionized FA (Figure 2C). Thus, FA added to the external buffer first bind at the outer leaflet of the vesicles, and quickly reach about 50% ionization, since the apparent pK_a of FA in the PC bilayer is 7.6 (Hamilton & Cistola, 1986). Some of the un-ionized species flip to the inner monolayer and then rapidly reach ionization equilibrium, thereby releasing protons at the inner monolayer of the vesicles, which diffuse into the inner volume containing pyranin. Since the internal volume of SUV's is very small, the effects of the binding of less than 1 mol % FA lead to a measurable decrease in internal pH (Kamp & Hamilton, 1992; see also Figure 6A below). When BSA is added to vesicles with cosonicated oleic

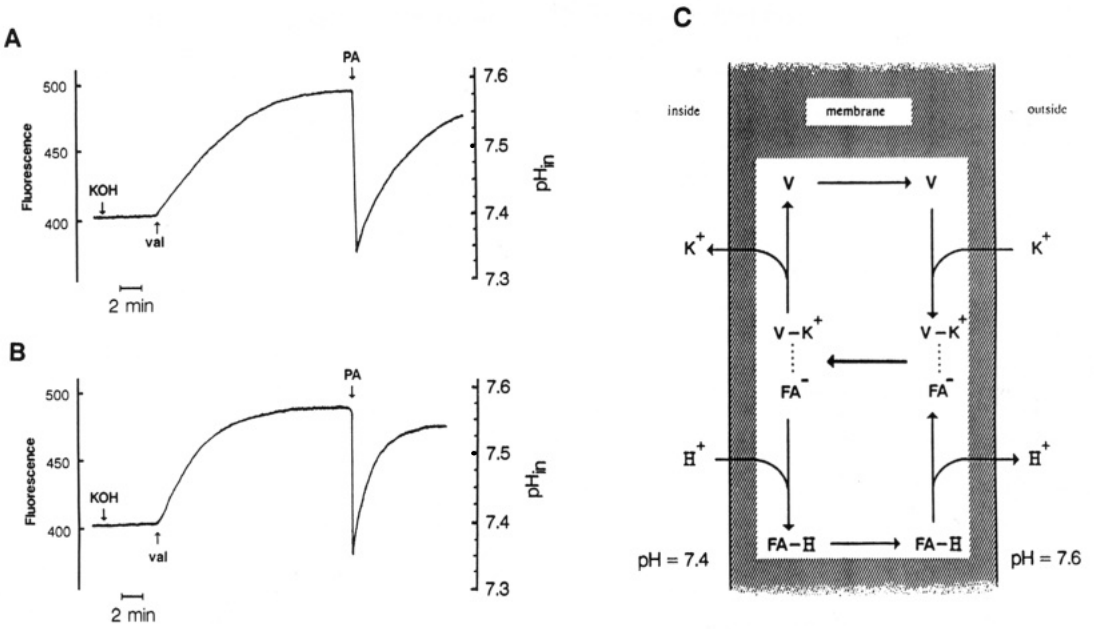


FIGURE 3: Slow transbilayer movement of ionized FA. (A) Vesicles (1440 nmol of PC) in 2 mL of buffer. First a pH gradient of 0.17 unit was generated by adding KOH. Proton leak was released by adding 2 nmol of valinomycin (val). Once the pH gradient had dissipated, 20 nmol of palmitic acid (PA) was added. This caused an instant acidification of the internal pH. The pH gradient dissipated at the same rate as in the first part of the experiment. (B) Experimental conditions as in (A), except that now 20 nmol of valinomycin was added. The dissipation of the pH gradient caused by palmitic acid was now much faster than prior to addition of FA. (C) Proposed mechanism for release of flip-flop of ionized FA by complex formation with valinomycin (V), with bound K^+, as indicated by the dotted line. The net result is that cyclic proton transfer by FA is accompanied by counterflow K^+ ions.

acid, FA are extracted from the outer leaflet via the aqueous phase. Since some FA in cosonicated vesicles reside in the inner leaflet, FA move to the outer leaflet to restore the concentration equilibrium in the bilayer. At this step protons are removed from the internal aqueous compartment, and pH_{in} increases. The pH_{in} increase (0.36 unit) was larger than the drop in pH_{in} in the former case (0.27 unit), even though the relative amounts (2 mol %) of oleic acid were equal in both cases (Figure 2A,B). This discrepancy can be explained by the suppressed ionization of FA at the inner leaflet when pH_{in} becomes acidic and the presence of less FA (un-ionized and ionized) in the inner leaflet at lower, compared to higher, pH.

Transbilayer Movement of FA in the Presence of Valinomycin. It is also possible that the changes in internal pH observed upon addition or extraction of FA are caused by fast transbilayer movement of the ionized, as opposed to the un-ionized, species. The pH changes would then be the result of independent proton flow balancing the charge movement of the ionized FA. This explanation can be excluded, since the events upon addition of FA to phospholipid bilayers (Figure 2) also take place in the presence of valinomycin, as illustrated in Figure 3A. In this experiment pH_{out} (initially 7.40) was first increased to 7.57 by adding KOH. Valinomycin (2 nmol) was added to abolish partially the diffusion potential and enhance the (background) proton leak. The pH gradient dissipated over a time course of minutes and was fit closely to an exponential decay ($k_b = 0.13 \pm 0.01$ min^{-1}). Once the pH had re-equilibrated across the bilayer ($pH_{in} = pH_{out} = 7.57$), palmitic acid (20 nmol, 1.4 mol % relative to PC) was added, causing the pH_{in} to drop instantly by about 0.23 unit, a decrease similar to that observed in the absence of valinomycin (Figure 2A). Subsequently, the pH gradient decayed exponentially (Figure 3A) with a rate constant slightly higher ($k_t = 0.15 \pm 0.01$ min^{-1}) than that found in the absence of added palmitic acid.

Rapid transbilayer movement of the palmitate anion can now be excluded for two reasons. First, if the ionized species moved faster than the un-ionized form in the experiment of Figure 3A, its movement would have been balanced by valinomycin-mediated K^+ flow, and the ionized FA at the inside would have bound protons to restore 50% ionization. Thus, upon addition of palmitic acid, an instantaneous alkalinization (or at least a smaller acidification than in the absence of valinomycin) should have occurred instead of the observed acidification. Second, proton leak was hardly enhanced by the presence of FA, indicating that cyclic proton transfer by FA, which must involve transbilayer movement of the ionized form, was very slow under these experimental conditions.

However, we observed enhanced transbilayer movement of the palmitate anion with larger amounts of valinomycin relative to FA. The experiment of Figure 3A was repeated with 1, 5, 10, and 20 nmol of valinomycin. The background proton leak increased with increasing valinomycin, demonstrating progressive collapse of the diffusion potential. We assumed that the background proton leak was saturated, or nearly so, at the highest amount of valinomycin investigated (2 mol % with respect to PC; Arents et al., 1981). With increasing amounts of added valinomycin the instantaneous acidification upon addition of PA was always the same, but we observed an increasingly faster decay of the pH gradient caused by the addition of PA, relative to the background proton leak. Figure 3B shows an experiment in which KOH was first added to generate a pH gradient, as in Figure 3A. Upon addition of 20 nmol of valinomycin, the pH gradient collapsed faster (exponentially, with $k_b = 0.24$ min^{-1}) than with 2 nmol of valinomycin ($k_b = 0.13$ min^{-1}). After equilibration of the outer and inner pH to 7.57, palmitic acid (20 nmol, a stoichiometric amount with respect to valinomycin) was added, and the pH_{in} dropped instantly 0.21 pH unit, as in Figure 3A. However, the pH gradient now collapsed much faster ($k_t = 0.64$ min^{-1}) than prior to the addition of palmitic acid. The pseudo-unimolecular rate constant for transbilayer movement of the ionized species [$k_{FA^-} = 1.78$ min^{-1} (0.029 s^{-1})]. Transbilayer movement of ionized FA is probably facilitated

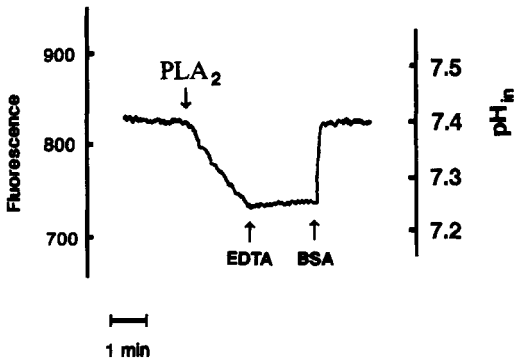


FIGURE 4: Effect of phospholipase A_2 activity on internal pH. To vesicles (2400 nmol of PC, in 2 mL of buffer) was added 0.05 unit of phospholipase A_2 (PLA_2) (with $CaCl_2$, final concentration ~0.01 mM). After a few minutes 1 mM EDTA was added, which stopped the phospholipase A_2 activity. Finally, the generated pH gradient was removed by addition of 10 nmol of BSA.

by the decrease in the diffusion potential. It is also possible that a complex between K^+ valinomycin and ionized FA moves across the membrane, as schematized in Figure 3C.

Transbilayer Movement of FA Generated by Phospholipase Activity. An alternative way to introduce FA into the bilayer of small unilamellar vesicles is by addition of phospholipase A_2 to the external buffer. Phospholipids in the outer leaflet will be hydrolyzed in the sn-2 position to produce lysophosphatidylcholine and FA. If some of the FA generated in the outer leaflet of SUV's move rapidly across the bilayer, acidification of the internal volume will be observed. Figure 4 depicts the results of an experiment in which phospholipase A_2 was added to PC vesicles with trapped pyranin. The pH_{in} dropped slowly with time, reflecting the slow, continuous production of FA.[2] When EDTA was added to scavenge the Ca^{2+} ions necessary for the phospholipase A_2 activity, the fluorescence no longer changed. The generated pH gradient was maintained over minutes (at least) and could be instantaneously reversed by addition of BSA to the vesicles (Figure 4).

Transbilayer Movement of Other FA and Lipophilic Acids. The transbilayer movement of native FA of different chain lengths and saturation was examined with the use of pyranin assay (in the absence of valinomycin). These FA included the medium-chain acid octanoic acid (8:0); the saturated series lauric (12:0), myristic (14:0), palmitic (16:0), and stearic (18:0) acids; and the unsaturated native FA oleic (18:1, cis), linoleic (18:2, cis,cis) and arachidonic (20:4, all cis) acids. We also examined two trans FA, elaidic (18:1) and linolelaidic (18:2, trans,trans) acids, which are produced in food processing and have been implicated in heart disease (Siguel & Leeman, 1993). It was found that all FA investigated equilibrated rapidly across the PC bilayer via the un-ionized form (Table I). Movement from the outer to inner monolayer was monitored by addition of aliquots of solutions of FA to the external buffer, and movement in the reverse direction by addition of BSA shortly after the drop in pH_{in} (Kamp & Hamilton, 1992; see also Figure 5A). Arachidonic acid showed quantitative binding to BSA, as expected (Savu et al., 1981). We also tested the ability of retinoic acid, a 20-carbon isoprenoid lipid with a 6-membered ring, to cross a PC bilayer. Retinoic acid has an apparent pK_a of ~7 in PC vesicles, and by analogy to FA, might be expected to exhibit rapid transbilayer movement via the un-ionized form (Noy, 1992). This hydrophobic acid produced a pH_{in} decrease similar to that observed for long-chain FA; the pH_{in} decrease was, however, not completely reversed by equimolar albumin (not shown). Thus, retinoic acid exhibits rapid transbilayer movement and weaker binding to BSA. Since retinoic acid is the most polar of the biologically prevalent retinoids, our results would predict an even faster transbilayer movement of retinol and retinal.

Properties of FA in model membranes and biomembranes are often studied by use of spin- or flourescent-labeling of the native species (Jost & Griffith, 1980). We used our assay to monitor the transbilayer movement of three commonly used FA analogoues: nitroxide-, anthroyloxyl-, and pyrene-labeled FA. For comparison, Figure 5A shows that 30 nmol (3.2 mol % relative to PC) of native stearic acid caused an instant acidification of the internal pH to pH_{in} 7.12, which was quantitatively reversed by addition of 10 nmol of BSA (FA:BSA = 3:1, mol/mol). Figure 5B shows that the nitroxide-labeled stearic acid 5-doxylstearic acid (30 nmol) caused the pH_{in} to drop to 7.0. The slightly larger pH decrease compared to native stearic acid suggests that the pK_a is lower for 5-doxylstearic acid, in accord with measurements by Miyazaki et al. (1992). Only a fraction of the doxylstearic acid could be extracted by the addition of a stoichiometric amount of BSA, as indicated by the failure to reverse the pH decrease completely (Figure 5B).

Two fluorescent-labeled FA were investigated: 1-pyrenenonanoic acid and 12-(9-anthroyloxy)stearic acid. Addition of 30 nmol (2.6 mol % relative to PC) of 1-pyrenenonanoic acid to SUV caused an instantaneous pH drop of 0.22 unit (Figure 5C), which could be reversed to a large extent by 10 nmol of BSA (FA:BSA = 3:1, mol/mol) and completely by equimolar BSA. Thus, the pyrene-labeled FA closely mimicked the behavior of native stearic acid in our assay. On the other hand, when 12-(9-anthroyloxy)stearic acid was added to vesicles (Figure 5D), the pH_{in} dropped to 7.15 slowly ($t_{1/2}$ = 1.2 min), in contrast to the immediate decrease in pH_{in} found for native stearic acid and the other FA analogues. The fact that pH_{in} decreased shows that the un-ionized form flip-flops faster than the ionized form (as for native FA). After addition of BSA, the pH_{in} increased slowly and only slightly, showing that BSA has a much lower binding affinity for 12-(9-anthroyloxy)stearic acid than the native FA. When an equimolar mixture of 12-(9-anthroyloxy)stearic acid with BSA (30 nmol each) was added to PC vesicles, the pH_{in} decreased to 7.20 with a rate (dotted line in Figure 5D) similar to that without BSA. The pseudo-unimolecular rate constant of transbilayer movement of the un-ionized 12-(9-anthroyloxy)-stearic acid k_{FAH}, calculated from the initial slope of the trace of Figure 5D (see Appendix), was 0.29 min^{-1} (4.8×10^{-3} s^{-1}). The results for all labeled FA are summarized in Table I.

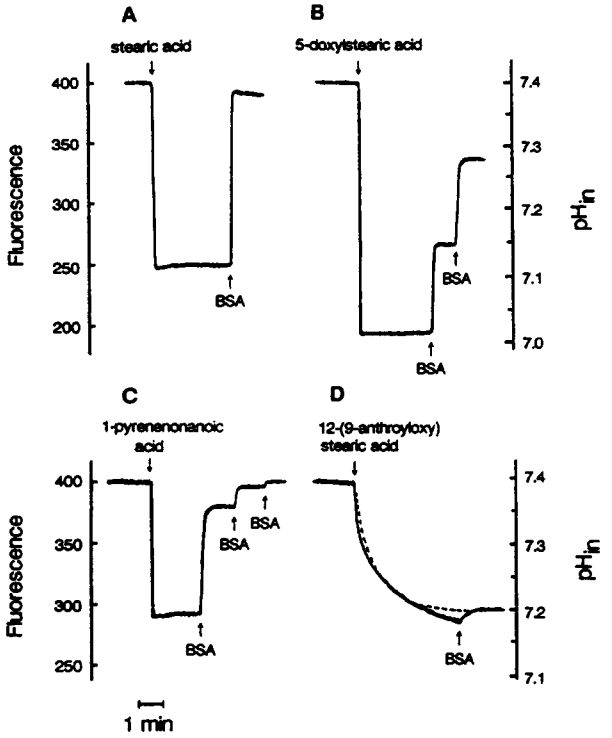
Distribution of FA between Vesicles, Albumin, and Water. The biological role of serum albumin is both to donate FA to, and extract FA from, membranes (Spector, 1986). The partitioning of FA depends on the affinities of the binding sites on albumin and membranes and on the relative occupancy of the high-affinity sites of BSA (Spector, 1986; Daniels et al., 1985). Our assay allowed the partition coefficient (molar fraction of FA bound to the PC membrane/molar fraction of FA bound to BSA) to be measured without the need to separate any of the species. We first studied palmitic acid, which has an extremely low solubility in water and a high affinity for phospholipid vesicles. Under the conditions used in the experiment the very small amount dissolved in the aqueous phase can be neglected (Anel et al., 1993). In an experiment

[2] In another experiment in which the amount of phospholipase A_2 was doubled, the continuous acidification of the internal volume was 2 times faster (not shown). This means that the rate of acidification is determined by the phospholipase A_2 activity and not by slow transbilayer movement of FA.

Table I: Transbilayer Movement of Fatty Acids and Lipophilic Acids: Half-Time ($t_{1/2}$) for Unfacilitated Movement (Flip-Flop) of Un-ionized Acid

	$t_{1/2} < 1$ s	$t_{1/2}$ measured	$t_{1/2} > 1$ h
fatty acids	[a]		
labeled fatty acids	5-doxylstearic acid 1-pyrenenonanoic acid	12-(9-anthroyloxy)stearic acid ($k_{FAH} = 4.8 \times 10^{-3}$ s^{-1})	
bile acids	deoxycholic acid chenodeoxycholic acid	cholic acid ($k_{CAH} = 0.056$ s^{-1})[b]	taurocholic acid taurodeoxycholic acid taurochenodeoxycholic acid
other acids	retinoic acid		

[a] Octanoic (8:0), lauric (12:0), myristic (14:0), palmitic (16:0), stearic (18:0), oleic (18:1, cis), elaidic (18:1, trans), linoleic (18:2, cis,cis), linolelaidic (18:2, trans, trans), arachidonic (20:4) acids. [b] $k_{CAH} = 0.033$ s^{-1} (in the presence of BSA).



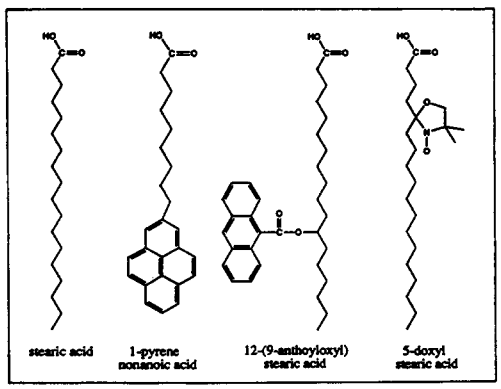


FIGURE 5: Transbilayer movement of labeled FA. In all experiments a stirred cuvette contained vesicles (950 nmol of PC) in 2.0 mL of buffer except for (C) where 1140 nmol of a different SUV preparation was used. (A) Addition of 30 nmol of stearic acid, followed by extraction with 10 nmol of BSA displayed a typical result of the FA assay. (B) Addition of 30 nmol of 5-doxystearic acid followed by first 10 nmol of BSA and subsequently 20 nmol of BSA. (C) Addition of 30 nmol of 1-pyrenenonanoic acid followed by three aliquots of 10 nmol of BSA. (D) Addition of 30 nmol of 12-(9-anthroyloxy)-stearic acid caused a slow decrease in internal pH which could not be reversed by 30 nmol BSA. The dotted line represents the decrease in pH_{in} upon addition of 30 nmol of BSA loaded with 30 nmol of 12-(9-anthroyloxy)stearic acid. Molecular structures are shown.

similar to that of Figure 2A, palmitic acid was added to vesicles in Hepes buffer and the change in fluorescence of trapped pyranin was recorded (Figure 6A). By repeating this protocol with different amounts of palmitic acid, a reproducible correlation between pH_{in} as a function of added FA was obtained (Figure 6B). Although essentially all the FA binds to the vesicle, the relationship is not linear because equivalent increments in FA produce smaller decreases in pH_{in} as a consequence of the altering ionization equilibrium of FA in the inner leaflet, which increasingly favors the un-ionized species at lower pH. The data points closely fit a calculated curve (dotted line in Figure 6B). The latter was found by calculating the expected drop in the pH_{in} upon binding of aliquots of FA, using the buffer equation for Hepes (pK_a = 7.55) and FA (apparent pK_a in the bilayer 7.6). For this calculation we assumed (i) the buffer concentration inside the vesicles to be 100 mM, (ii) an equimolar distribution of un-ionized FA between the inner and outer leaflets, and (iii) no flip-flop of the ionized species. The calculations were carried out for different vesicle diameters, taking the membrane thickness to be 4.0 nm and the surface area per PC molecule to be 0.7 nm^2 (Small, 1967). An internal volume of 0.25 μL/μmol of PC, corresponding to a vesicle diameter of 20.5 nm, gave the best fit (shown). This was slightly smaller than the calculated internal volume of 0.40 μL/μmol of PC, based on a vesicle diameter of 25 nm of sonicated vesicles (Kornberg & McConnell, 1971). However, the calculated diameter is in good agreement with the mean diameter of 22.3 nm (SD = 5.3 nm) determined by electron microscopy of our samples.

The partitioning of palmitic acid between BSA and vesicles was monitored by the pyranin fluorescence as shown in Figure 6C. BSA (15 nmol) was first added to vesicles, and a small increase in pH_{in} was observed, reflecting extraction of endogeneous FA in the vesicles. Extrapolating from Figure 6B, we estimated that ~1.5 nmol of FA (~0.12 mol % with respect to PC) was now bound to BSA (1 mol of FA/10 mol of BSA), and we assumed that no FA remained in the vesicle bilayer. Subsequently, 15-nmol aliquots of aqueous palmitic acid were added to the aqueous mixture of BSA and vesicles, and incremental decreases in pH_{in} were observed. In contrast to Figure 6A, subsequent aliquots of FA caused larger decrements in pH_{in}. From Figure 6B the amount of FA bound to the vesicles was determined, and the remaining amount was assumed to be bound to BSA (neglecting the very small amount of FA dissolved in water).

The experiment of Figure 6C was repeated (not shown) with lower proportions of BSA (1.5 and 5 nmol). These results, together with those of Figure 6C, can be represented in terms of the calculated partition coefficients versus the relative amount of palmitic acid bound to BSA (Figure 6D). The partition coefficient depends on the relative occupancy of the high-affinity sites on BSA, not on the absolute amounts of PC and BSA. These plots show that, with increasing palmitic acid, nearly all the FA binds to BSA until a mole ratio of 3 mol of FA/mol of BSA is reached and then most of the added palmitic acid binds to the vesicles.

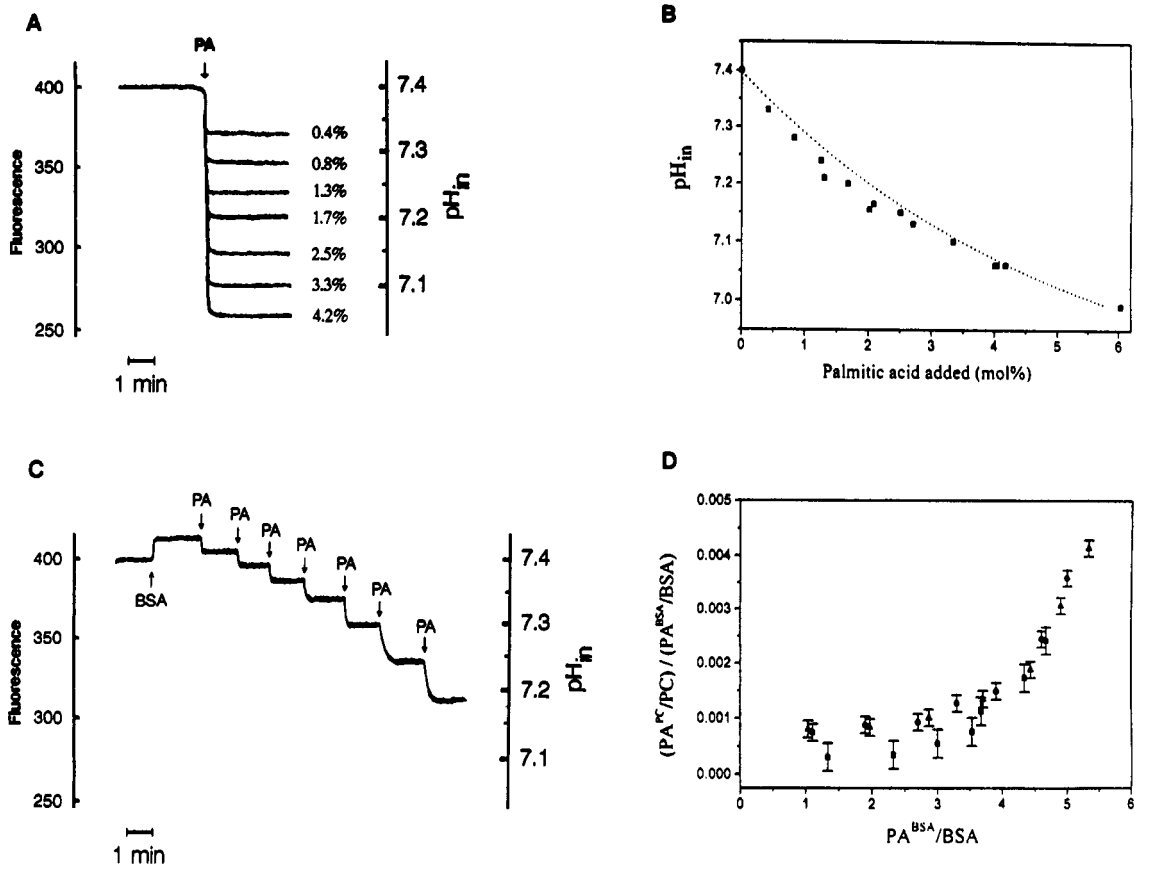


FIGURE 6: Partitioning of palmitic acid between vesicles and BSA. (A) Increasing amounts (5, 10, 15, 20, 30, 40, and 50 nmol) of aqueous palmitic acid (PA) were added to vesicles (1200 nmol of PC). pH_{in} dropped instantaneously, to a larger extent with increasing amounts of FA. (B) Since the pH gradients generated upon the addition of palmitic acid decay very slowly in the absence of valinomycin, a reproducible correlation between the drop in internal pH and the mole percent FA added to the membrane was found. The data points were collected from three different vesicle preparations. The dotted line represents the calculated change in pH_{in} (see text). (C) To vesicles (1200 nmol of PC) in 2 mol of buffer was added BSA (15 nmol) prior to the addition of seven aliquots of 15 nmol of aqueous palmitic acid (PA). (D) Partition coefficient versus the total amount of PA added (relative to BSA) for three experiments; 1.5 nmol of BSA (■); 5.0 nmol of BSA (●); 15 nmol of BSA (▲).

Using the protocols described for palmitic acid, we determined the partitioning of oleic acid and three shorter chain FA (myristic, lauric, and octanoic acids). Figure 7A summarizes results obtained from additions of different amounts of FA to vesicle suspensions without BSA present (as in Figure 6A). Oleic acid gave results indistinguishable from those for palmitic acid, as did myristic acid. In contrast, comparable amounts of lauric acid caused smaller decreases in pH_{in}, although the trend was similar to that of long-chain FA. Octanoic acid produced a very small change in pH_{in}. We conclude that palmitic, oleic, and myristic acids partition into the PC vesicles under our conditions, and the very small amount of FA in water is not detected. The data for lauric acid and octanoic acid reflect a lower affinity for PC and greater partitioning into water.

The partitioning of lauric acid between the aqueous phase and vesicles was determined as follows. The pH_{in} changes caused by the four additions of lauric acid to the vesicles (Figure 7A) were compared with those caused by palmitic acid (dashed line in Figure 7A), which binds completely to vesicles, and the fraction of lauric acid bound to the vesicles was estimated using the same pK_a for lauric acid as for palmitic acid in PC vesicles (7.6).[3] The remaining amount was assumed to be dissolved in water. The amount of lauric acid bound to PC was proportional to the amount dissolved in water (Figure 7B), the result expected for nonsaturating binding. The partition coefficient (wt % lauric acid bound to PC/wt % lauric acid in water) calculated from the slope of the line connecting the data points was 1700 (Table II). The shortest chain fatty acid tested, octanoic acid, displayed weak binding to the vesicles. A small decrease in pH_{in} was found only when much larger amounts were added. The partition coefficient for octanoic acid, determined in the same way as for lauric acid, was 23 wt %/wt % (Table II).

A comparison of partitioning of palmitic, oleic, and lauric acids between vesicles and BSA was determined from experiments analogous to those carried out in Figure 6B. The partition coefficients were calculated as above. The results for oleic acid and palmitic acid (Figure 7C) are very similar. A similar experiment was done with lauric acid, although the amount of lauric acid dissolved in water could not be neglected (Figure 7B). Again, 15 nmol of BSA was first added to vesicles and a small amount of endogeneous FA extracted. Upon the first two additions of 15 nmol of lauric acid, no decreases in pH_{in} were observed, indicating that all added FA was bound to BSA. After the subsequent additions of lauric acid, decreases in pH_{in} were found and the amounts of lauric acid bound to PC estimated from the pH_{in} decreases found for palmitic acid (Figure 7A). The amount dissolved in the water was calculated by using the measured partitioning of lauric acid between vesicles and water (Figure 7B). The remaining fraction of lauric acid was assumed to be bound to BSA. The partitioning of lauric acid between BSA and water (Figure 7B) as well as the partition coefficients of lauric acid between vesicles and BSA (Figure 7C) was plotted. The results were indicative of 3 high-affinity, 2–3 intermediate-affinity, and

[3] Unpublished results in this laboratory.

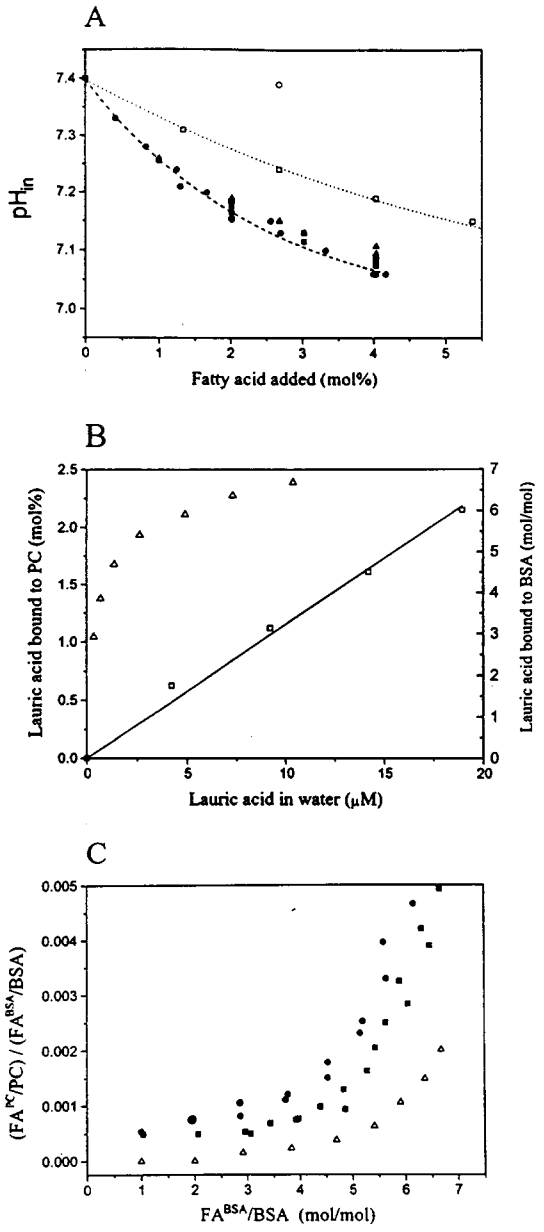


FIGURE 7: Distribution of palmitic, oleic, myristic, lauric, and octanoic acids among vesicles, water, and BSA. (A) To several preparations of SUVs with trapped pyranin were added aliquots of palmitic acid (●), oleic acid (■), myristic acid (▲), lauric acid (□), and octanoic acid (○), and the change in internal pH was recorded. The PC concentration was known for every experiment and was usually approximately 1 μmol of PC in 2 mL of buffer. The relationship between the total FA added (mol % relative to PC) and pH_{in} was identical for oleic, palmitic, and myristic acids (dashed line) but different for lauric acid (dotted line). (B) Distribution of lauric acid between vesicles and water (□), and between BSA and water (△). (C) Results of several titrations as in Figure 6C: partitioning of palmitic acid between 990 nmol of PC vesicles and 15 nmol of BSA or between 1120 nmol of PC vesicles and 15 nmol of BSA (●); partitioning of oleic acid between 990 nmol of PC vesicles and 15 nmol of BSA or between 2560 nmol of PC vesicles and 100 nmol of BSA, (■); partitioning of lauric acid between 1120 nmol of PC vesicles and 15 nmol of BSA (△).

an unspecified amount of low-affinity binding sites for lauric acid on BSA, as found for palmitic and oleic acids.

Transbilayer Movement and Partition Coefficients of Bile Acids. Bile acids are another important group of biological lipophilic acids, and we used our assay to assess the ability of several bile acids to cross small unilamellar vesicles spontaneously. Results for cholic acid (CA) are presented in Figure 8A. Addition of aqueous sodium-cholate (200 nmol) to vesicles

Table II: Partition Coefficients for Fatty Acids and Bile Acids

molecule	wt % bound to PC/wt % in water	wt % bound to BSA/wt % in water
octanoic acid (C8:0)	1700	
lauric acid (C12:0)	23	
cholic acid	187	165
deoxycholic acid	667	869

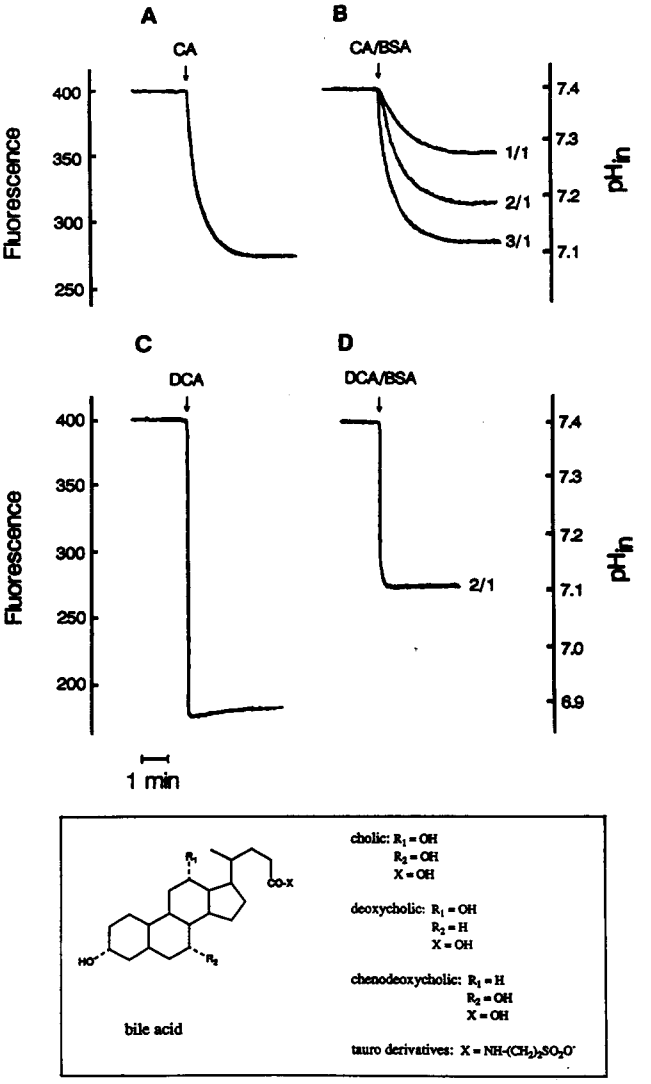


FIGURE 8: Vesicles with trapped pyranin (1080 nmol of PC) in 2 mL of buffer. (A) Addition of 200 nmol of sodium cholate (CA). (B) Three experiments: addition of 100 nmol of BSA loaded with 100, 200, and 300 nmol of cholate. (C) Addition of 200 nmol of sodium deoxycholate (DCA). (D) Addition of 200 nmol of sodium deoxycholate loaded on 100 nmol of BSA. Molecular structures are shown.

(initial $pH_{in} = pH_{out} = 7.40$) caused pH_{in} to drop to 7.10 over a period of minutes ($t_{1/2} = 12$ s). The generated pH gradient then remained for minutes, showing that the CA did not disrupt the vesicles and did not enhance proton leak. In comparison with long-chain FA, the pH decrease occurred slowly; additionally, much more bile acid had to be added to the vesicles to induce a comparable drop in pH_{in} (similar to the case of octanoic acid). Assuming that the apparent pK_a of CA in a PC vesicle is 7.3 in the outer leaflet and 6.8 in the inner leaflet, and that the distribution of un-ionized CA between the outer and inner leaflets is 2:1 (Cabral et al., 1986), we calculated that 14.3 nmol of the added 200 nmol partitioned to the vesicles. Thus, the partition coefficient (wt % cholate bound to the PC vesicles/wt % cholate dissolved in water) was 187 (Table II), within a factor of 2 of the value of 100 reported

previously (Cabral & Small, 1989). With the proportion of CA in the vesicles estimated as above, the pseudo-unimolecular constant for transbilayer movement of the un-ionized species (k_{CAH}) calculated from the initial slope (see Appendix) was k_{CAH} = 3.36 min^{-1} (0.056 s^{-1}). Thus, CA spontaneously crosses a PC bilayer via the un-ionized species, but much slower than FA.

Experiments with CA in the presence of BSA (CA:BSA mole ratios of 1:1, 2:1, and 3:1) also showed a slow decrease in pH_{in} after each addition (Figure 8B). The final pH_{in} values were used to calculate the amount of CA bound to the vesicles, as for lauric acid. From the calculated partition coefficient in the absence of BSA, the aqueous concentration of cholate was calculated, and the remaining fraction was assumed to be bound to BSA. For all initial mole ratios of CA to BSA it was found that ~35% of the added cholate was bound to BSA, ~60% was dissolved in the aqueous phase, and ~5% was partitioned to the PC bilayer. The partition coefficient with respect to BSA (wt % CA bound to BSA/wt % CA dissolved in water) was 165 (Table II). Having evaluated the amount of cholate bound to the PC vesicles and assuming that the dissociation from BSA and the binding to PC were fast, the pseudo-unimolecular rate constant for transbilayer movement of un-ionized cholic acid, k_{CAH}, could be calculated (see Appendix) from the initial slopes of the traces in Figure 8B. The values for k_{CAH} (2.0 ± 0.2 min^{-1}, 0.033 s^{-1}) were independent of the initial CA:BSA ratio, but were slightly slower than that found when aqueous cholate was added in the absence of albumin (k_{CAH} = 3.36 min^{-1}, 0.056 s^{-1}).

Deoxycholic acid (DCA) and chenodeoxycholic acid (CDCA) showed a different behavior than CA when added to vesicles. DCA produced an instantaneous drop in pH_{in} and a larger decrease in pH_{in} compared to an equivalent amount of added cholate (Figure 8C). Thus, DCA (i) flips much faster than CA and (ii) partitions into the membrane to a greater extent than CA. From an experiment with a smaller amount of DCA (100 nmol; results not shown), the partition coefficient (evaluated as for cholate) was found to be 667 (wt % DCA in membrane/wt % DCA in water; Table II). CDCA gave results similar to those for deoxycholate (not shown). The higher partitioning of DCA and CDCA, compared to CA, into vesicles is expected on the basis of their hydrophobic index (Heuman, 1989). The taurine conjugates of the above three bile acids were also investigated to determine their ability to cross the PC vesicle bilayer. Each conjugated bile acid caused a small decrease in fluorescence (the equivalent of ~0.03 pH_{in} unit) with the addition of 300 nmol, but no further effects were observed with up to 900 nmol. Because these bile acids are more hydrophilic than their unconjugated forms and will partition less favorably into PC vesicles, we added higher concentrations. However, the hydrophobicity index of taurodeoxycholate is the same as chenodeoxycholate and only slightly lower than that of un-ionized CDCA (Heuman, 1989). Therefore, the partitioning of taurodeoxycholate into vesicles, even a lower concentration, should be sufficient to yield a decrease in pH_{in} were flip-flop to occur. We conclude that the conjugated acids did not exhibit flip-flop.

The partitioning of DCA among vesicles, BSA, and water was determined by adding DCA (200 nmol) mixed with BSA (100 nmol) to the vesicle suspension (Figure 8D). The internal pH dropped instantaneously to 7.10. Using Figure 6B it was calculated that 14.3 nmol of DCA was bound to the vesicles. Taking the partition coefficient of DCA between the membrane and water to be 667, it was calculated that 45.7 nmol of DCA was dissolved in the water phase, and the remaining 140.0 nmol of DCA was assumed to be bound to BSA. The partition coefficient (wt % DCA on BSA/wt % DCA dissolved in water) was calculated to be 869 (Table II), higher than that for cholate.

DISCUSSION

The pyranin fluorescence assay (Kamp & Hamilton, 1992) is a simple and direct approach for monitoring the transbilayer movement of molecules with titrating group(s). This study, which examined a number of FA and other lipophilic acids in the presence and absence of ionophores and albumin, supports our previous conclusion that FA move across a phospholipid bilayer spontaneously and rapidly via the un-ionized (i.e., unprotonated and uncharged) form (see Appendix, diagram 3). These experiments have provided unequivocal demonstration that (i) native FA can flip-flop across a phospholipid bilayer rapidly and (ii) unidirectional transbilayer movement of FA gives rise to a pH gradient, which persists because of the slow proton leak across the bilayer. Furthermore, our results for other native lipophilic acids and covalently labeled FA show the above mechanism to be a general one. These qualitative conclusions are reinforced by the quantitative agreement of the observed decreases in pH_{in} with calculated decreases (Figure 6B). Table I summarizes our findings of all investigated compounds.

Rapid transbilayer movement across phospholipid bilayers of the uncharged form compared to the charge form is a typical feature of lipophilic acids (Prestegard et al., 1979; Cabral et al., 1986, 1987; Eastman et al., 1991; Kamp & Hamilton, 1992). Recent studies of the kinetics of vesicle fusion suggest rapid redistribution (within 1 s) of oleic acid across the bilayer of large unilamellar vesicles in response to an imposed pH gradient (Wilschut et al., 1992) in accord with our hypothesis. In model membranes, flip-flop of the charged form of FA is probably limited by the fast buildup of a diffusion potential following the generation of the pH gradient. Our experiments show that increasing amounts of valinomycin, which diminish the diffusion potential and release the background proton leak, also enhance flip-flop of ionized FA. When a stoichiometric amount of valinomycin relative to FA is present, significant cyclic proton transfer by FA is observed, which must involve flip-flop of the ionized form. It is possible that a complex of K^+–valinomycin with ionized FA crosses the membrane. Consistent with this proposal, other researchers have provided evidence for interactions of lipophilic and organic anions with cation complexes of valinomycin (Feinstein & Felsenfeld, 1971; Davis & Tosteson, 1975; Marinetti et al., 1978).

Comparison of several covalently modified FA by the pyranin assay revealed important differences in their transbilayer movement and binding to albumin. Except for the 12-(9-anthroyloxy)stearic acid, all modified FA showed very rapid flip-flop (Table I). Although it was previously suggested that ionized 12-(9-anthroyloxy)stearic acid moves faster than the un-ionized form (Storch & Kleinfeld, 1986), we found the opposite, in agreement with all the other acids studied. However, the rate constant from our assay ($k_{FAH} = 4.8 \times 10^{-3}$ s^{-1}; Table I) is close to that determined ($k = 2.3 \times 10^{-3}$ s^{-1}) from the assay of Kleinfeld and Storch (1993), which monitored transfer of 12-(9-anthroyloxy)stearic acid between vesicles under conditions of pH and temperature similar to those in this study. A similar type of measurement with pyrene–FA suggested fast transbilayer movement compared to transfer between vesicles (Doody et al., 1980). These divergent results can now be rationalized by our studies, which constitute a more direct measure of transbilayer movement and show that pyrene–FA moves rapidly compared to 12-(9-anthroyloxy)stearic acid. The slow rate of the movement

of 12-(9-anthroyloxy)stearic acid across PC vesicles has been used to advance the argument that proteins are necessary for transport of FA across membranes (Storch & Kleinfeld, 1986; Kleinfeld & Storch, 1993). Our results show that the rate of transbilayer movement of the 12-(9-anthroyloxy)stearic acid is at least 150 times slower than that of native FA.

Each of the labeled FA showed different partitioning between vesicles and albumin, a reflection of different affinities of BSA for the ligands. The pyrene-labeled FA showed the highest affinity binding (similar to native FA under the experimental conditions), 5-doxylstearic acid a significantly lower affinity, and the 12-(9-anthroyloxy)stearic acid an apparently much lower affinity. The 5-doxylstearic acid was been used to examine the binding of FA to albumin (Ge et al., 1990). This study shows that BSA has a much lower affinity for this probe than for native stearic acid. In general, the preceding results illustrate the risks in using a modified FA to model the behavior of native FA and show that one specific property may be marked appropriately, while another property may not be.

The reproducible correlation between the mole percent of added long-chain FA and the decrease in pH_{in} enabled us to investigate quantitatively partitioning of different FA between BSA and vesicles as well as partitioning of medium-chain FA (octanoic and lauric acids) between water and vesicles. From partitioning data (Figure 7C) we found three high-affinity sites for oleic, palmitic, and lauric acids on BSA, in agreement with previous studies (Spectro et al., 1986; Hamilton, 1992). The partition coefficients for those high-affinity sites (Figure 7C) were similar (within a factor of 2) to those reported by Daniels et al. (1985) for partitioning of myristic acid between DMPC bilayers and BSA and by Noy et al. (1986) for partitioning of palmitic acid between egg PC and BSA. Compared to long-chain FA, lauric acid showed decreased partitioning to PC vesicles (Figure 7C). The binding affinity of lauric acid to both BSA (Spector et al., 1969) and PC bilayers (Anel et al., 1993) is lower than that for longer chain FA such as palmitic acid. The partitioning results for lauric acid show that the relative decrease in the binding affinity is larger for PC than BSA. The decreased partitioning of shorter chain FA into phospholipid bilayers (in the presence or absence of albumin) can explain the diminished effects of these FA relative to long-chain FA on membrane-related processes (Philipson, 1984; Philipson & Ward, 1985). Similarly, effects of bile acids on membranes are determined by their partitioning into membranes rather than their absolute concentration (Lichtenberg, 1985; Shoemaker & Nichols, 1992). Our studies showed smaller effects on the pH_{in} of SUV's from unconjugated bile acids compared to the same concentration of long-chain FA. This is explained by measurements of partitioning among vesicles, BSA, and water, which revealed a much lower affinity of PC bilayers and BSA for bile acids compared to FA with >12 carbons (Table II).

The use of different bile acids in our experiments permitted a comparison of the rates of transbilayer movement of related molecules with different hydrophobicities (Table I). Three broad categories of movement were found: very slow ($t_{1/2}$ > 1 h), very fast ($t_{1/2}$ < 1 s), and intermediate (Table I). Cholic acid exhibited a measurable flip rate (k_{CAH} = 0.056 s^{-1}; $t_{1/2}$ = 12 s), whereas the more hydrophobic deoxy- and chenodeoxycholic acids (Heuman, 1989) exhibited fast flip ($t_{1/2}$ < 1 s; Table I), at least 10 times faster than CA. Measurements of pseudo-unimolecular rate constants in PC vesicles at pH 3.0 have shown a 30-fold slower rate for CA compared to DCA (Cabral et al., 1987), consistent with the above results at pH ~7.4. NMR data yield a greater (50 times) rate constant for cholic acid than that measured in the present experiment, even after correction is made for the lower amount of un-ionized CA in the vesicle interface at pH 7.4 vs pH 3.0. This disparity suggests that there are interactions between bile acid and bile salt in the phospholipid bilayer at pH 7.4 that impede the movement of cholic acid across the bilayer and that decreased pH may greatly facilitate spontaneous movement of CA across membranes. Our studies suggest that passive diffusion is a possible mechanism for CDCA and DCA, even at pH ~7.4.

Bile acids with the strongly acidic taurine group ($pK_a \approx 1$ in micelles; Hofmann & Small, 1967) showed no transbilayer movement over a time scale of minutes. Even if the apparent pK_a were slightly higher in the PC vesicles, the amount of un-ionized acid at pH 7.4 would be extremely small, and the rate would be expected to be very slow, analogous to the case of phosphatidic acid (Eastman et al., 1991). A ^{13}C NMR spectroscopic approach also detected no movement of taurine conjugates of bile acids across PC vesicles (Cabral, 1987). The majority of conjugated bile acids are not passively absorbed as they pass through the biliary tree or small intestinal tract but are actively transported across membranes of the terminal ileum (Hofmann, 1984). The mechanism for CA absorption in tissues has not been clearly elucidated. In the liver, where pH values are similar to those in the present study, movement across the plasma membrane appears to be governed by both a carrier process and a low-affinity, high-capacity process, which could be simple diffusion (Veith et al., 1992; Calflisch et al., 1990). CA uptake in liver cells is associated with intracellular acidification (Vieth et al., 1992), which can be explained by the mechanism proposed herein.

The use of the enzyme phospholipase A_2 permitted introduction of FA into the phospholipid bilayer from within the bilayer, in contrast to our general protocol of addition of FA in the external buffer. Under the conditions of the experiment, with limited hydrolysis, the phospholipase A_2 will act only on the outer leaflet of the vesicle bilayer (Bhamidipati & Hamilton, 1989). The two product molecules, which together have a shape very similar to that of the phospholipid molecule, will initially be present in the outer leaflet, and the surface pressure changes will be smaller than those following insertion of FA into the outer leaflet of vesicles via addition to the external buffer. Nevertheless, FA produced by phospholipase A_2 moved instantaneously to the inner leaflet. Therefore, it can be concluded that the fast flip-flop of FA in PC bilayers is not caused by newly created surface pressure differences between the inner and outer leaflets.

The movement of FA across a phospholipid bilayer following phospholipase A_2 hydrolysis has implications for cell physiology. In normal physiological conditions phospholipase A_2 hydrolyzes membrane phospholipids to provide important biologically active FA, such as arachidonic acid. These FA can act as messengers in signal transduction (Hannigan & Williams, 1991) and are precursors for prostaglandin synthesis, and the question of whether they can move rapidly to the opposite leaflet of the membrane is important in understanding their biological effects. Our results show that FA produced by PLA_2 hydrolysis of egg PC, which include arachidonic acid, can rapidly reach the opposite leaflet of the membrane. We further showed that arachidonic acid added externally to PC vesicles exhibited fast flip-flop (Table I). Under abnormal physiological conditions, such as ischemia, PLA_2 activity is greatly increased (Hazen et al., 1991). The rapid equilibration of significant amounts of PLA_2-produced FA could create pH gradients across membranes, and such pH gradients could contribute to cellular damage. FA added to cardiac sarcolemal

vesicles at levels estimated to reflect those produced in cardiac ischemia (~5 mol % with respect to membrane phospholipid) have been shown to stimulate Na^+/Ca^{2+} exchange and increase Ca^{2+} permeability (Philipson, 1984; Philipson & Ward, 1985). Whether such electrophysiological effects are due to H^+ gradients may now be tested.

CONCLUSIONS

Demonstration of rapid, unfacilitated movement of FA across phospholipid bilayers makes this a potentially viable mechanism in biological membranes. This study specifically removes several theoretical objections to the notion that FA can move spontaneously across membranes at a rate sufficient for cellular utilization, such as the argument that FA cannot cross a lipid bilayer because they are fully negatively charged (Potter et al., 1989). Thus, the need for a transport protein cannot be based on the assumption that a phospholipid bilayer necessarily constitutes a barrier to transbilayer movement of FA. On the other hand, the present data [(and those of Kamp and Hamilton (1992)] with model membranes cannot be interpreted to mean that passive diffusion is an exclusive or universal mechanism in biomembranes. It is possible that the structure of some biological membranes may prevent the spontaneous transbilayer movement of FA. For example, the lipid bilayer may not be directly accessible to FA because of a high concentration of membrane proteins and glycolipids, and a FA-binding protein may be required to sequester the FA to the membrane bilayer, where it can undergo flip-flop (Veerkamp et al., 1991). If a FA can reach one leaflet of the membrane bilayer, a transport protein would have to compete with the phospholipid bilayer, which has a high capacity for both binding and transport of FA. On the basis of our studies, a potential function of a transmembrane transporter protein, as suggested previously (Skulachev, 1991), would be to move *ionized* FA across the membrane in response to the unfacilitated movement of the un-ionized FA and the production of a transmembrane pH gradient. Valinomycin may serve as a model FA transporter. While our studies lend more credence to the passive diffusion mechanism, it is clear that the issue of transport mechanism(s) of FA or bile acids in biomembranes must be examined on a case by case basis.

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APPENDIX: KINETICS OF FATTY ACID-MEDIATED PROTON MOVEMENT ACROSS SMALL UNILAMELLAR VESICLES

Background Proton Leak. When a pH gradient is imposed upon SUV prior to any addition of FA, for instance by adding KOH to the external medium (Figure 3B), we assume the proton flux across the membrane (J_H) to be equal to an unspecific (background) proton leak, J_H^b (significant only in the presence of valinomycin). At the small pH gradients applied J_H^b is proportional to the pH gradient across the SUV according to the following phenomenological relationship (Arents et al., 1981; Westerhoff & Van Dam, 1987; Kamp, 1991):

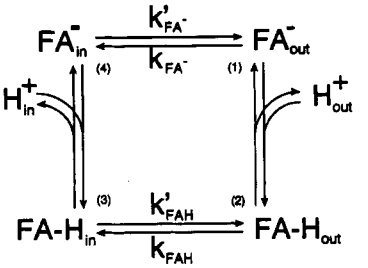
$$J_H^b = L(pH_{out} - pH_{in}) \quad (1)$$

J_H^b is related to the change in internal pH as follows:

$$J_H^b = J_H = B_{in}\, d(pH_{in})/dt \quad [nmol/(min, \mu mol\ of\ PC)] \quad (2)$$

where B_{in} is the internal buffer capacity, which can be calculated to be approximately constant over the narrow pH ranges in our experiments [B_{in} = 21 nmol of H^+/(pH unit, μmol of PC), for vesicles of 25-nm diameter containing 100 mM Hepes buffer in the entrapped aqueous volume]. Since the external pH is strongly buffered, one finds by integrating that eqs 1 and 2 predict the internal pH to increase exponentially toward pH_{out} with a relaxation rate constant k_b ($=L/B_{in}$).

Fatty Acid-Mediated Proton Leak. When a pH gradient is generated instantaneously upon the addition of an aliquot of N mol of FA (per mol of PC) to the external buffer (Figure 3B), the subsequent slow proton efflux will equal the background leak plus the rate at which protons are transferred by the FA. The latter involves transbilayer movement of both un-ionized and ionized FA. We apply the following kinetic diagram for the flip-flop and (de)protonation reactions of FA in a membrane:



(3)

We assume that the protonation and deprotonation reactions are extremely fast, so that at either side of the membrane the protonated and unprotonated forms of FA are always at equilibrium; i.e., the following buffer equations apply at all times:

$$pH_{in} = pK_a - \log[p(FAH_{in})/p(FA^-_{in})] \quad (4)$$

$$pH_{out} = pK_a - \log[p(FAH_{out})/p(FA^-_{out})] \quad (5)$$

where $p(X)$ denotes the probability of the FA to be in state X.

$p(FAH_{out})$, $p(FA^-_{out})$, $p(FAH_{in})$, and $p(FA^-_{in})$ can be expressed in terms of pH_{in} and pH_{out} by combining expressions 4 and 5 with the assumptions that (i) the sum of the probabilities of all four states is 1, (ii) at the time scale of our proton efflux experiments, the transmembrane distribution of FAH corresponds to the equilibrium distribution (i.e., $1/k_{FAH}$ and $1/k'_{FAH}$ are much shorter than the time scale of observation), and (iii) flip-flop of un-ionized FA is equally fast in both directions ($k_{FAH} = k'_{FAH}$). As a consequence

$$p(FA^-_{out}) = 1/(1 + 2 \times 10^{(pK_a - pH_{out})} + 10^{(pH_{in} - pH_{out})}) \quad (6)$$

$$p(FA^-_{in}) = 10^{(pH_{in} - pH_{out})}/(1 + 2 \times 10^{(pK_a - pH_{out})} + 10^{(pH_{in} - pH_{out})}) \quad (7)$$

$$p(FAH_{in}) = p(FAH_{out}) = 10^{(pK_a - pH_{out})}/(1 + 2 \times 10^{(pK_a - pH_{out})} + 10^{(pH_{in} - pH_{out})}) \quad (8)$$

If the internal pH is lower than the external pH (Figure 3B), $p(FA^-_{in})$ will be smaller than $p(FA^-_{out})$, causing a net transmembrane flow of FA^- through the transition $1 \rightleftharpoons 4$ of diagram 3, J_{14}. Since all the other transitions are fast, this will cause net proton movement across the membrane, mediated by FA, J_H^{FA}. We will now derive how J_H^{FA} is related to J_{14}. Let for an infinitesimal duration δt an amount of $\delta\xi$ flow through this transition. As the actual flow occurs, this will have decreased the amount of FA^-_{out} by $\delta\xi$. Because of the near equilibrium of transitions $4 \rightleftharpoons 3$, $3 \rightleftharpoons 2$, and $2 \rightleftharpoons 1$, this decrease will immediately redistribute over all the forms of the fatty acid, such that, for instance

$$\delta_1 p(FAH_{in})/\delta\xi = -10^{(pK_a - pH_{out})}/(1 + 2 \times 10^{(pK_a - pH_{out})} + 10^{(pH_{in} - pH_{out})}) \quad (9)$$

Immediately after the transition, FA^-_{in} will have increased by $\delta\xi$, but also this increase will redistribute over all the forms of the FA, such that, using eqs 4 and 7, for instance

$$\delta_2 p(FAH_{in})/\delta\xi = +10^{(pK_a - pH_{out})}/(1 + 2 \times 10^{(pK_a - pH_{out})} + 10^{(pH_{in} + \delta pH_{in} - pH_{out})}) \quad (10)$$

where it should be noted that the internal pH may have changed by δpH_{in}. Because of the transition $4 \rightleftharpoons 3$, protons have been taken up from the internal volume:

$$-\delta H^+_{FA}/(N\,\delta\xi) = 1 + [\delta p(FAH_{in})/\delta\xi + \delta p(FAH_{out})/\delta\xi + \delta p(FA^-_{out})/\delta\xi - \delta p(FA^-_{in})/\delta\xi] \quad (11)$$

where the subscript FA to δH^+ stresses that this is the number of protons binding to the fatty acid. Using that

$$\lim_{\delta\xi \to 0} (\delta pH_{in}) = 0 \quad (12)$$