Pyranine (8-Hydroxy-1,3,6-pyrenetrisulfonate) as a Probe of Internal Aqueous Hydrogen Ion Concentration in Phospholipid Vesicles†

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ABSTRACT: The fluorescence intensity (at 510 nm) of the hydrophilic pyrene analogue 8-hydroxy-1,3,6-pyrenetrisulfonate (pyranine) is strongly dependent upon the degree of ionization of the 8-hydroxyl group (pK_a = 7.2) and hence upon the medium pH, over the range pH 6–10. Because of its polyanionic character, pyranine does not bind significantly to phospholipid vesicles having a net anionic surface charge. As a result, it is possible to form vesicles in the presence of pyranine which, after removal of external probe by gel filtration, contain pyranine entrapped within the internal aqueous compartment. Once entrapped, pyranine does not readily leak out of the vesicles. Because the fluorescence properties of entrapped pyranine resemble closely the properties of bulk pyranine solution with respect to pH sensitivity, pyranine can be used as a reliable reporter of aqueous pH changes within anionic vesicles. When HCl is rapidly added to a suspension of unilamellar soybean phospholipid (asolectin) vesicles preincubated at alkaline pH, a biphasic decrease in the pH of the vesicle inner aqueous compartment is observed. An initial, very rapid and electrically uncompensated H^+ influx ($t_{1/2}$ < 1 s) results in the generation of a transmembrane electric potential opposing further H^+ influx. This leads to the development of a much slower ($t_{1/2} \cong 5$ min), valinomycin-sensitive, proton–counterion exchange which continues until the proton concentration gradient is eliminated. Similar results were obtained in asolectin vesicles prepared by detergent dilution, in sonicated egg phosphatidylcholine vesicles, and in multilamellar asolectin liposomes. The rather high permeability of soybean lipid membranes to H^+ is surprising in view of the widespread use of these lipids for the reconstitution of membrane proteins which are thought to generate or utilize H^+ ion gradients in energy transduction reactions.

Differences in hydrogen ion concentration across biological membranes play an important intermediary role in a number of cellular processes, including oxidative and photosynthetic phosphorylation and solute transport. In isolated organelles such as mitochondria and chloroplasts, these gradients can be quantified by using techniques based upon the pH-dependent distribution of radioactive or fluorescent amines of appropriate pK_a (Rottenberg, 1979). However, the methods utilized to measure the distribution of radioactively labeled amines make detailed kinetic investigations extremely difficult, while studies with pH indicator dyes and fluorescent amines are complicated by membrane–probe interactions which vary with experimental conditions. Furthermore, neither technique possesses sufficient sensitivity to allow application to reconstituted systems, where the volume of the internal aqueous compartment defined by the artificial phospholipid vesicle is generally very much smaller than the internal volumes typical of organelles (Watts et al., 1978).

Recently, Kano & Fendler (1978) introduced the use of 8-hydroxy-1,3,6-pyrenetrisulfonate (pyranine)[1] as a pH-sensitive fluorescence probe for steady-state measurements of the aqueous interior of neutral and anionic artificial vesicular systems. We have extended this technique to allow continuous measurements of internal hydrogen ion concentration in artificial phospholipid vesicles of the type often used in reconstitution studies (Racker, 1973). In this paper we report a preliminary characterization of passive hydrogen ion fluxes across unilamellar and multilamellar phospholipid vesicle membranes.

Experimental Methods

Partially purified soybean phospholipids (asolectin; Associated Concentrates, Woodside, NY) were further purified by acetone and ether extractions (Kagawa & Racker, 1971). Egg phosphatidylcholine (Sigma Chemical Co.) was used without further purification. Sonicated unilamellar vesicles were formed by evaporating under a stream of nitrogen a chloroform solution of the phospholipids onto the walls of a small glass test tube. A small volume (usually 1 mL) of a suspension buffer containing 0.1 M KCl, 0.2 M sucrose, 5 mM tricine/KOH, 5 mM Mes/KOH, and 0.5 mM pyranine (pH 8.2) was added, and the mixture was sonicated under N_2 in a bath type sonicator (Laboratory Supplies Co., Hicksville, NY) until clear (~60 min). Care was taken to maintain the mixture at pH 8.2–8.3 throughout the sonication by adding microliter aliquots of KOH as necessary. Multilamellar liposomes were prepared by vortexing the dried lipids for 30 s in the suspension buffer. Unilamellar cholate dilution vesicles (Racker et al., 1975) were prepared by resuspending 20 mg of dried lipid in 0.5 mL of suspension buffer containing 1.4% cholate using a brief sonication in the bath sonicator. An aliquot of the lipid solution was then diluted 50-fold into the suspension buffer (minus cholate) to induce vesicle formation. Vesicles containing pyranine were separated from external aqueous pyranine on a Sephadex G-25 column (0.6 × 19 cm). The final phospholipid concentration of the pooled vesicle fractions was determined as the inorganic phosphate content by using the procedure of Ames (1966).

Fluorescence was measured in a 1-cm cuvette which was stirred continuously in a thermostated holder maintained at 25 °C. The exciting light was defined by a 100-W tungsten lamp (Oriel) and a 460-nm interference filter. The fluorescence emission, which was channeled through a 12 in. long

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[1] Abbreviations used: pyranine, 8-hydroxy-1,3,6-pyrenetrisulfonate; DMPC, dimyristoylphosphatidylcholine; tricine, *N*-tris(hydroxymethyl)methylglycine; Mes, 2-(*N*-morpholino)ethanesulfonic acid.

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Table I: Binding of 8-Hydroxy-1,3,6-pyrenetrisulfonate (Pyranine) to Phospholipid Vesicles[a]

phospholipid	pyranine present[b]	relative fluorescence intensity[c]
asolectin	inside and outside	100
asolectin	outside only	9
asolectin plus dicetyl phosphate	outside only	6
DMPC	inside and outside	100
DMPC	outside only	34
DMPC plus dicetyl phosphate	outside only	4

[a] Unilamellar vesicles were prepared by sonication as described under Experimental Methods. The final phospholipid concentration in the 3-mL samples was 95–168 μg/mL. Dicetyl phosphate was present at a molar ratio of 1 per 4 phospholipids when added. [b] "Inside and outside" means pyranine was present during the sonication and that external aqueous pyranine was removed by Sephadex G-25 filtration. "Outside only" means vesicles were prepared in the absence of pyranine, after which pyranine (0.5 mM) was added to the external buffer before passage through the Sephadex column. [c] Fluorescence was excited at 460 nm and measured at 520 nm. The solution pH was 8.2. Fluorescence intensities were normalized to account for variations in phospholipid concentration.

fiber optic bundle (0.25-in. diameter) and a 520-nm interference filter, was detected with an RCA photomultiplier tube. Changes in fluorescence intensity were recorded on a strip-chart recorder. Changes in external medium pH were monitored by a Sargent miniature combination pH electrode connected to a Digiphase pH meter.

Valinomycin was dissolved in ethanol. When added, the final concentration of ethanol in the reaction mixture was ≤0.5%.

Results

Properties of Vesicle-Entrapped Pyranine. Kano & Fendler (1978) observed that ionization of the 8-hydroxyl group of pyranine at alkaline pH (pK_a = 7.2) was associated with a pronounced red shift in the fluorescence excitation maximum, from 400 (pH 4) to 450 nm (pH 10), while the 510-nm emission maximum remained essentially unchanged. The amount of 510-nm fluorescence excited at 450 nm, therefore, reflects the concentration of the unprotonated species and hence the medium pH.

The pyranine polyanion is very hydrophilic, and binds only slightly to vesicle membranes having a net anionic surface charge (Table I). Membranes comprised of zwitterionic lipids such as DMPC bind somewhat higher levels of the probe, although this binding can be largely eliminated by incorporating an anionic lipid such as dicetyl phosphate into the bilayer. Forming vesicles in the presence of pyranine results in the incorporation of significant levels of the probe into the vesicle fraction after gel filtration, indicating that some pyranine molecules are bound to or become entrapped within the vesicles. The low polarization values for the fluorescence from pyranine molecules associated with the vesicle fraction (p = 0.05, 25 °C; Clement & Gould, 1980) and the insensitivity of this polarization to large changes in membrane fluidity (not shown), together with the binding data presented in Table I, strongly suggest that pyranine molecules associated with the vesicle fraction are free in solution in the internal aqueous phase and are not bound to the phospholipid bilayer.

Pyranine entrapped in unilamellar asolectin vesicles exhibits fluorescence properties very similar to those reported by Kano & Fendler (1978) for pyranine in solution, except for a very slight alkaline shift in the pK_a (Figure 1), possibly resulting from the proximity of negatively charged phospholipids on the inner surface of the bilayer. When a small volume of HCl is rapidly injected into a suspension of unilamellar vesicles preequilibrated at alkaline pH, a biphasic decrease in the

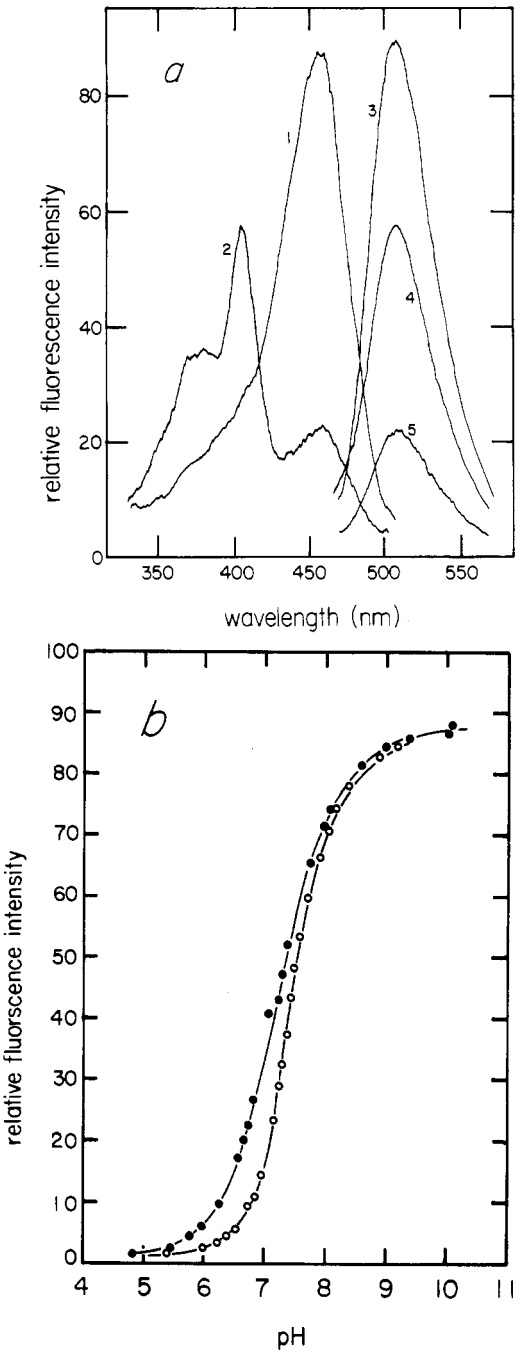

FIGURE 1: pH-dependent changes in the fluorescence intensity of pyranine entrapped within unilamellar phospholipid vesicles. (Panel a) Fluorescence excitation and emission spectra for asolectin vesicles (750 μg of phospholipid) containing entrapped pyranine. Vesicles were suspended in 3 mL of 0.2 M sucrose, 0.1 M KCl, 5 mM tricine/KOH, and 5 mM Mes/KOH at 20 °C. Excitation spectra (emission λ = 510 nm) were measured at pH 8.3 (trace 1) and pH 6.6 (trace 2). Emission spectra [excitation λ = 460 nm (traces 3 and 5) or 405 nm (trace 4)] were also measured at pH 8.3 (trace 3) and pH 6.6 (traces 4 and 5). (Panel b) Variation of the fluorescence intensity (excitation λ = 460 nm; emission λ = 520 nm) of 1 μM pyranine (●) or pyranine-containing asolectin vesicles (○; 150 μg of phospholipid) in 3 mL of 0.2 M sucrose, 0.1 M KCl, 5 mM tricine/KOH, and 5 mM Mes/KOH. The vesicle sample contained 5 μM valinomycin and 200 nM gramicidin to facilitate transmembrane H^+ equilibration.

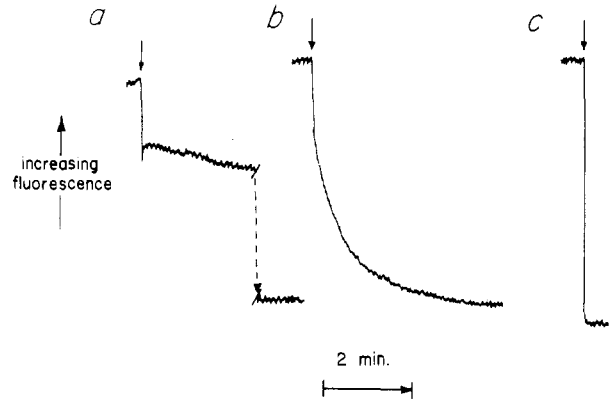

FIGURE 2: Changes in the fluorescence intensity of pyranine entrapped in unilamellar asolectin vesicles following a rapid decrease in external pH. The 3-mL sample contained 0.2 M sucrose, 0.1 M KCl, 5 mM Mes/KOH, 5 mM tricine/KOH (pH 7.8), and vesicles containing 464 μg of phospholipid. The downward arrow indicates the addition of 12 μL of 1 N HCl, which lowered the external pH to 6.2. (Trace a) Asolectin vesicles alone; (trace b) valinomycin (1.6 μg) was added to the vesicle suspension 2 min before the HCl pulse; (trace c) Triton X-100 (final concentration 0.03%) was added to the vesicle suspension 2 min before the HCl pulse.

fluorescence intensity of the entrapped pyranine is observed (Figure 2). An initial fast kinetic component, limited in these studies by the mixing time ($t_{1/2} \sim 1$ s), is followed by a much slower decrease in fluorescence intensity to a new constant level (Figure 2). Lysing the vesicles with Triton X-100 prior to the addition of HCl resulted in a very fast, monophasic change in fluorescence intensity corresponding to the change in pH of the external buffer, indicating that the slow kinetic component observed in the absence of detergent can most likely be attributed to passive diffusion of protons across the bilayer into the vesicle interior. The acceleration of the slow kinetic component by valinomycin in the presence of K^+ indicates that the rate limitation for proton diffusion into the vesicle during this phase is probably electrically compensating counterion redistributions.

No difference in the fluorescence response was noted when the external pH of the vesicle suspension was lowered by the addition of HCl, H_2SO_4, or a small volume of a concentrated Mes solution preadjusted to give the appropriate final pH. A very similar pattern of fluorescence changes, of opposite direction, was also observed in vesicle suspensions equilibrated at mildly acidic pH and pulsed with NaOH (data not shown).

There are a number of possible explanations for the biphasic kinetics of the pyranine fluorescence decrease observed after a rapid change in external pH. For example, such a response would be expected if a significant proportion of the probe molecules were present outside of the vesicles (but see Table I) or if a portion of the vesicle population were very leaky to protons and counterions. In either case, the relative proportions of the total fluorescence change contributed by the fast and slow kinetic component should remain constant, regardless of the initial and final pH. This was not found to be the case, however (Figure 3). When the external pH was rapidly changed from 8.2 to 7.5 (ΔpH = 0.7), the fast component comprised ~60% of the total fluorescence decrease. However, when the external pH was changed from 7.2 to 6.5 (ΔpH = 0.7), the fast component comprised only ~20% of the total fluorescence decrease.

These data suggest that the fast component of the fluorescence decrease actually represents an initial rapid, and at least partially electrically uncompensated, diffusion of protons into the vesicles. This uncompensated charge influx would lead to the formation of a transmembrane electric po-

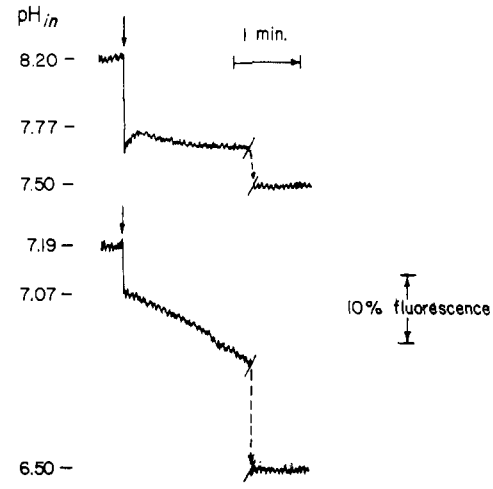

FIGURE 3: Relative contributions of fast and slow kinetic components of the fluorescence decrease of vesicle-entrapped pyranine following a rapid drop in external pH. (Upper trace) Asolectin vesicles (464 μg of phospholipid) were equilibrated in 3 mL of a medium containing 0.2 M sucrose, 0.1 M KCl, 5 mM Mes/KOH, and 5 mM tricine/KOH (pH 8.2) before addition of 6 μL of 0.1 N HCl (arrow), which lowered the external pH to 7.5. (Lower trace) Conditions were the same as in the upper trace, except that the initial pH was 7.19 and the final pH after the HCl pulse was 6.5. Values for pH_{in} were calculated from the relative fluorescence intensity by using Figure 1.

tential (inside positive) which eventually would slow further proton influx to a counterion-limited proton–counterion exchange. That is, at the end of the fast component of the fluorescence decrease, the negative free energy of the proton concentration gradient (ΔG_c), given by

$$\Delta G_c = RT \ln ([H^+_{in}]/[H^+_{out}]) \quad (1)$$

where $[H^+_{in}]$ and $[H^+_{out}]$ represent the concentrations of protons inside and outside the vesicles, respectively, becomes equal to the positive free energy of the opposing electric potential (ΔG_e), given by

$$\Delta G_e = ZF\Delta\psi \quad (2)$$

where Z is the net uncompensated charge per proton and $\Delta\psi$ is the electric potential in volts. Since each proton moving into a vesicle carries a +1 charge, and if a constant internal buffering capacity over the range of the pH transition is assumed,[2] ΔG_e should be linearly proportional to the *change* in internal proton concentration ($\Delta[H^+_{in}]$)

$$\Delta G_e = k\Delta[H^+_{in}] \quad (3)$$

Similarly, ΔG_c can be expressed in terms of $\Delta[H^+_{in}]$ since, for an increase in the initial internal H^+ concentration ($[H^+_{in}]^i$)

$$[H^+_{in}] = [H^+_{in}]^i + \Delta[H^+_{in}] \quad (4)$$

Substituting, eq 1 can be rewritten as

$$\Delta G_c = RT \ln \frac{[H^+_{in}]^i + \Delta[H^+_{in}]}{[H^+_{out}]} \quad (5)$$

Plots of ΔG_c as a function of $\Delta[H^+_{in}]$ for the experiment shown in Figure 3 are presented in Figure 4. Since an estimate for ΔG_c (or $-\Delta G_e$) can be obtained from the apparent

[2] Titrations of asolectin vesicle suspensions (in the presence of gramicidin plus valinomycin to facilitate transmembrane H^+ equilibration) indicated a relatively constant buffering capacity over the pH range 8.5–6.5. Entrapped tricine and Mes contributed <10% of the total internal buffering capacity in this region, assuming a distribution of membrane buffering residues between the outer and inner monolayers of 2:1 (Yeagle et al., 1976).

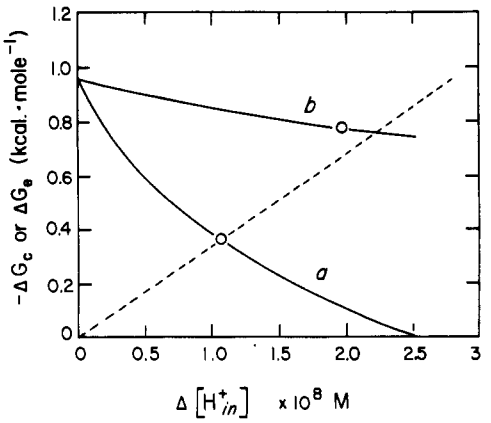

FIGURE 4: Changes in free energy of proton concentration gradient (ΔG_c) and transmembrane electric potential (ΔG_e) with changes in internal hydrogen ion concentration ($\Delta[H^+_{in}]$) for the experiment shown in Figure 3. The solid lines indicate the change in $-\Delta G_c$ (calculated from eq 5) for pH 8.2 → 7.5 (curve a) and pH 7.19 → 6.5 (curve b) transitions. The dashed line represents the values for ΔG_e (calculated from eq 3), assuming $k = 3.4 \times 10^{10}$ cal·L/mol^2. The circles (O) represent values for $-\Delta G_c$ calculated from the internal pH at the end of the fast kinetic component (Figure 3) according to eq 1.

$[H^+_{in}]$ at the end of the fast kinetic component by using eq 1 (see Figure 3), an approximation for k in eq 3 can be made. The calculated value for ΔG_c at the end of the fast kinetic component for the pH 8.2 → 7.5 transition is −370 cal·mol^{-1}. If this value is assumed to be correct, a plot of ΔG_e as a function of $\Delta[H^+_{in}]$ (eq 3) can also be obtained, yielding a value for k of 3.4×10^{10} cal·L/mol^2 (Figure 4).[3] Such a plot predicts the intercept between the ΔG_c curve [for the pH 7.2 → 6.5 transition (Figure 3)] and the ΔG_e curve to occur at $\Delta G_c = -760$ cal·mol^{-1}, in very good agreement with the calculated value of −775 cal·mol^{-1} obtained from Figure 3 by using eq 1.

The conditions for the experiment presented in Figure 3 were chosen so that the initial value for ΔG_c was the same for each pH transition, although for a given incremental change in ΔG_c 10 times as many protons would enter the vesicles during a pH 7.2 → 6.5 transition as during a pH 8.2 → 7.5 transition, resulting in a much faster buildup of $\Delta\psi$ in the former case. The same conclusions were obtained in a different fashion in the experiment shown in Figure 5, in which $[H^+_{in}]^i$ was held constant and the amount of HCl added in the pulse, and hence $[H^+_{out}]$, was varied. Once again, very good agreement was obtained between the intercepts of the ΔG_c (calculated from eq 5) and ΔG_e (calculated from eq 3; $k = 3.4 \times 10^{10}$ cal·L/mol^2) curves and the values for ΔG_c calculated from the apparent internal pH at the end of the fast component of the fluorescence decrease by using eq 1.

Taken together, these data indicate that, under the conditions employed here, the initial fast decrease in the fluorescence of asolectin vesicle entrapped pyranine following an acidification of the external medium represents a rapid, electrically uncompensated influx of protons into the vesicles. This conclusion is consistent with the fact that valinomycin accelerated the slow component of the fluorescence response (Figure 2) and suggests that the noninstrumental "overshoot" often seen

[3] It is theoretically possible to calculate the proportionality constant k from the vesicle internal volume, the membrane specific capacitance, and the internal buffering capacity. While reasonable approximations for the former values can be made, a quantitative estimate of the buffering contribution of the membrane inner surface is difficult to construct in the absence of information on the content and distribution of buffering groups within the bilayer.

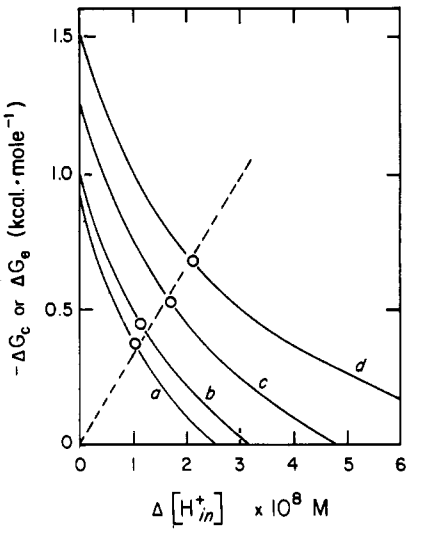

FIGURE 5: Changes in free energy of proton concentration gradient (ΔG_c) and transmembrane electric potential (ΔG_e) with changes in internal hydrogen ion concentration. Reaction conditions were essentially as described in the legend to Figure 3 except that the initial pH was 8.2 in all cases. The amount of HCl added was varied to give final external pH values of 7.5 (curve a), 7.42 (curve b), 7.24 (curve c), or 7.06 (curve d). The dashed line represents the values for ΔG_e (calculated from eq 3) assuming $k = 3.4 \times 10^{10}$ cal·L/mol^2. The circles represent values for $-\Delta G_c$ calculated from the internal pH at the end of the fast kinetic component of the fluorescence decrease according to eq 1.

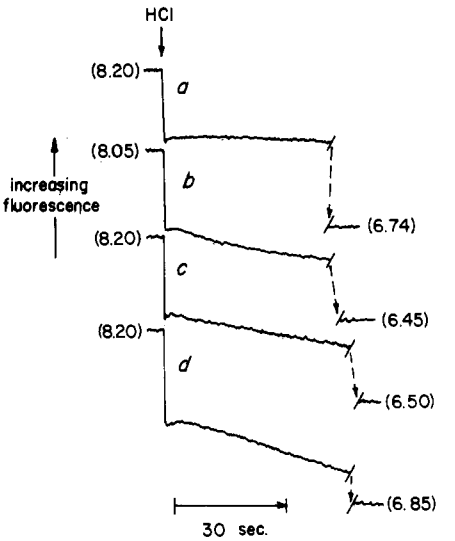

FIGURE 6: Changes in fluorescence intensity of pyranine entrapped in vesicles and liposomes. (Trace a) Unilamellar asolectin vesicles prepared by sonication; (trace b) unilamellar asolectin vesicles prepared by cholate dilution; (trace c) multilamellar asolectin liposomes; (trace d) unilamellar egg phosphatidylcholine vesicles prepared by sonication. Reaction conditions were as described in the legend to Figure 2 for trace a. Initial and final pH values are given in parentheses.

at the end of the fast phase (Figures 2 and 3) represents a transient hyperpolarization of the membrane, resulting in a brief proton efflux prior to the slower, counterion-limited proton influx.

Very similar patterns for the pyranine fluorescence response were also obtained in sonicated vesicles comprised of egg phosphatidylcholine, in unilamellar asolectin vesicles prepared by cholate dilution, and in large, multilamellar liposomes made from asolectin (Figure 6). In each case, although there was some variation in the rate of the slower, counterion-limited portion of the proton influx, a biphasic fluorescence response was observed.

Discussion

Pyranine has been found to be a reliable and convenient probe of the internal aqueous compartment of anionic unilamellar phospholipid vesicles. The hydrophilic molecule does not bind significantly to anionic membranes and, once entrapped within the vesicles, does not readily leak out (Kano & Fendler, 1978). These properties, along with the pronounced sensitivity of pyranine's fluorescence intensity to changes in hydrogen ion concentration, make this probe suitable for investigations of passive and facilitated proton and counterion fluxes in vesicular membrane systems. Furthermore, with this probe it is possible to continuously measure, in real time, the intravesicular pH, allowing kinetic resolution of transmembrane proton transport which has heretofore not been possible in small unilamellar vesicles.

An unexpected finding was the biphasic kinetics for the acidification of the internal aqueous compartment following a rapid drop in external pH. The appearance of the slower kinetic component appears to be the result of a transmembrane diffusion potential resulting from the rapid and (at least partially) electrically uncompensated initial influx of hydrogen ions into the vesicle. The number of hydrogen ions which must enter each vesicle in order to achieve pH equilibration is determined by the internal buffering capacity of the vesicles, which appears to be rather high in asolectin vesicles around pH 7, probably due to the presence of a small amount of phosphatidic acid in the bilayer. The relatively small internal volume of these vesicles (Watts et al., 1978) means that a large amount of counterion redistribution could lead to significant changes in internal counterion concentration, so that further counterion fluxes, driven by the electric potential, would be against a counterion concentration gradient. This effect would tend to magnify the positive free energy contribution of the electric potential to passive H^+ influx, although the extent of any counterion concentration gradient formed under the conditions of this study is unknown.

The nature of the mobile counterions(s) in these experiments is also not known with certainty, although preliminary studies suggest that, even in the absence of valinomycin, the primary counterion movement accompanying H^+ influx is probably K^+ efflux, rather than Cl^- influx (Clement & Gould, 1981). The actual counterion(s) moving will undoubtedly vary with the ionic composition of the medium, however.

Finally, one of the most interesting findings in the preliminary characterization of transmembrane ion movements presented here is the unexpectedly high permeability of small unilamellar asolectin vesicles to hydrogen ions relative to other monovalent ions. Very similar results were recently obtained by Nichols et al. (1980) and Nichols & Deamer (1980) for large unilamellar egg phosphatidylcholine vesicles, suggesting that a relatively high proton permeability may be an intrinsic property of curved membrane bilayers. While the presence of a highly active proton-conducting contaminant within the asolectin and egg phosphatidylcholine preparations used in these studies cannot be completely excluded as a possible explanation for the observed high proton permeability of these membranes, this does not seem likely since very similar results have been obtained with chromatographically pure preparations of synthetic lipids such as dimyristoylphosphatidylcholine (N. R. Clement and J. M. Gould, unpublished experiments). Nor can the rapid proton permeation be attributed to electroneutral fluxes of HCl, since biphasic changes in internal pH very much like those reported here were observed for vesicles suspended in K_2HPO_4 and pulsed with H_2SO_4 (Clement & Gould, 1981). The observed high proton conductivity of the small unilamellar asolectin vesicles is nevertheless surprising in view of the fact that a large variety of membrane proteins which generate or utilize transmembrane proton gradients as energy-rich intermediate states have been successfully reconstituted in these vesicles. Although the factors contributing to a successful reconstitution are complex, it may be that the relatively poor permeability of asolectin vesicles to counterions and the resulting propensity to maintain transmembrane electric potentials are important factors in the reconstituted activities of certain membrane proteins.

Acknowledgments

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