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Kinetics of Hydrogen Ion Diffusion across Phospholipid Vesicle Membranes†

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ABSTRACT: The membrane-impermeant, pH-sensitive fluorescence probe 8-hydroxy-1,3,6-pyrenetrisulfonate can be entrapped within the internal aqueous compartment of unilamellar phospholipid vesicles, where it serves as a reliable indicator of internal aqueous hydrogen ion concentration [Clement, N. R., & Gould, J. M. (1981) *Biochemistry 20*, 1534-1539]. When the external (medium) pH of a suspension of soybean phospholipid vesicles was rapidly changed from 8.2 to 6.65, the rate of subsequent H^+ influx into the vesicles, measured as the change in pyranine fluorescence, was limited (in KCl media) by the rate of charge-compensating counterion redistributions. The half-time for the pyranine fluorescence change (corresponding to an internal pH change from 8.2 to 7.43), which was several minutes in the absence of valinomycin, could be decreased to ~300 ms, but not further, by the K^+ ionophore valinomycin. Proton ionophores such as gramicidin or bis(hexafluoroacetonyl)acetone (1799), on the other hand, decreased the time required for transmembrane H^+ equilibration to <1 ms. These findings indicate that the intrinsic permeability of unilamellar vesicle membranes to hydrogen ions is surprisingly high and much greater than the observed permeabilities of other small ions.

The maintenance and regulation of ion gradients across biological membranes are crucial factors in proper cellular function. The need for specific electrolyte imbalances in such varied functions as nerve impulse transmission, enzyme function, and osmotic balance has long been established. In recent years, primarily as a result of the theories of Mitchell (1968), the central role of transmembrane hydrogen ion fluxes in cellular energetics has also become recognized.

By themselves, phospholipid bilayer membranes are generally considered to be relatively impermeable to most ions, including protons. However, several recent studies of hydrogen ion movements across unilamellar phospholipid vesicle membranes have led to the conclusion that these membranes are several orders of magnitude more permeable to protons and/or hydroxyl ions than to other small, monovalent ions (Nichols et al., 1980; Nichols & Deamer, 1980; Clement & Gould, 1981a), with a net H^+/OH^- permeability of $\sim 10^{-4}$ cm/s, compared with permeabilities of 10^{-10}-10^{-14} cm/s for other monovalent cations.

Utilizing a hydrophilic, pH-sensitive fluorescence probe trapped within the inner aqueous compartment of unilamellar vesicles, Clement & Gould (1981a) were able to directly monitor (in real time) changes in intravesicular pH following an abrupt change in the pH of the external medium. In that study, it was found that transmembrane H^+ equilibration was complete in <1 s when electrically compensating counterion fluxes were enhanced with valinomycin plus K^+. Unfortunately, those experiments were limited in their kinetic resolution by the sample mixing time, so that transmembrane pH equilibrations occurring in <1 s were not resolved.

In this paper we report the results of experiments similar to those of Clement & Gould (1981a) but performed by utilizing a rapid-mixing procedure in order to allow millisecond time resolution of the transmembrane proton fluxes.

Experimental Methods

Preparation of Vesicles. Unilamellar vesicles were prepared from purified (Kagawa & Racker, 1971) soy phosphatides (asolectin,[1] Associated Concentrates, Woodside, NY) by a

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[1] Abbreviations used: asolectin, purified soybean phospholipids; Tricine, *N*-tris(hydroxymethyl)methylglycine; Mes, 2-(*N*-morpholino)-ethanesulfonic acid; pyranine, 8-hydroxy-1,3,6-pyrenetrisulfonate; DMPC, L-α-dimyristoylphosphatidylcholine; 1799, bis(hexafluoroacetonyl)acetone; DLPC, L-α-dilaurylphosphatidylcholine; DPPC, L-α-dipalmitoylphosphatidylcholine.

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sonication procedure as described earlier (Clement & Gould, 1981a). Vesicles were prepared at room temperature in 0.1 M KCl, 5 mM Tricine/KOH, and 5 mM Mes/KOH (pH 8.2) containing 2.5 mM 8-hydroxy-1,3,6-pyrenetrisulfonate (pyranine, lasar grade from Eastman Chemical Co.), and were separated from external (untrapped) pyranine by gel filtration on Sephadex G-25. For some experiments vesicles were prepared from synthetic dimyristoylphosphatidylcholine (DMPC, Sigma Chemical Co.) containing various amounts of egg phosphatidic acid (Sigma) by a similar procedure, except that the sonication step and all subsequent steps were performed at 35 °C in order to prevent vesicle fusion (Kantor & Prestegard, 1975; Schullery et al., 1980). The phospholipid concentration of the final vesicle suspension was determined by the method of Ames (1966).

Measurement of Transmembrane Proton Fluxes. Hydrogen ion diffusion into phospholipid vesicles was monitored by the fluorescence technique described in detail by Clement & Gould (1981a). This procedure is based upon the entrapment of the pH-sensitive hydrophilic fluorescence probe pyranine within the internal aqueous compartment of the vesicle, where the probe molecule remains free in solution (Clement & Gould, 1980, 1981a). Changes in the internal hydrogen ion concentration (i.e., changes in fluorescence intensity) are monitored following an abrupt change in the pH of the external medium. In the experiments reported here, two different methods for changing the external pH were employed.

Stopped-Flow Rapid-Mixing Measurements. The two sample drive syringes of a Durrum-Gibson D-110 stopped-flow spectrofluorometer were loaded with (1) a suspension of asolectin vesicles in buffer (pH 8.2) and (2) a solution of an identical buffer (minus vesicles) which had been adjusted to a lower pH, so that the final pH of a 1:1 mixture with the vesicle solution was 6.65. The fluorescence of vesicle-entrapped pyranine[2] was excited by a 150-W stabilized xenon arc lamp through a grating monochomator (peak excitation wavelength = 460 nm). Fluorescence emission was detected through a Corning 3-69 cutoff filter by a Hamamatsu R378 end window photomultiplier tube. The signal was processed through a Durrum photometric log amplifier and monitored on a Tektronix storage oscilloscope. Data from the oscilloscope display were recorded by photographing the screen with an oscilloscope camera. When reaction half-times exceeded 60 s, the change in fluorescence was recorded on a Heath-Schlumberger strip chart recorder. The temperature of the sample syringes, mixing chamber, and observation cuvette was maintained at 25 °C with a constant-temperature circulating water bath.

Conventional Mixing Acid Pulse Measurements. An aliquot of the vesicle preparation in buffer was placed in a 1-cm plastic fluorescence cuvette, which was held in a thermostated jacket and stirred continuously by a small magnetic stirring bar. Pyranine fluorescence was excited with 460-nm light from a 100-W tungsten lamp (Oriel) defined by a grating monochromator (Oriel). Fluorescence emission was detected through a 12 in. long ($^1/_4$ in. diameter) fiber optic bundle and a 520-nm interference filter (10-nm bandwidth; Oriel) by an RCA 1P-28 photomultiplier tube connected through a Schoeffel M-460 picoammeter to a Heath-Schlumberger chart recorder. Hydrochloric acid sufficient to change the sample pH from 8.2 to 6.65 was added in a small volume (<15 μL) to the sample, and the change in fluorescence intensity with time was recorded. Changes in external medium pH were monitored by a Sargent miniature combination pH electrode connected to a Digiphase pH meter. All experiments were performed at 25 °C unless otherwise noted.

[2] The pyranine concentration *inside* the vesicles was assumed to be the same as the concentration present during sonication (2.5 mM). The "bulk" concentration of pyranine in the vesicle suspension, following the removal of the nonincorporated probe by gel filtration, was ~100 μM.

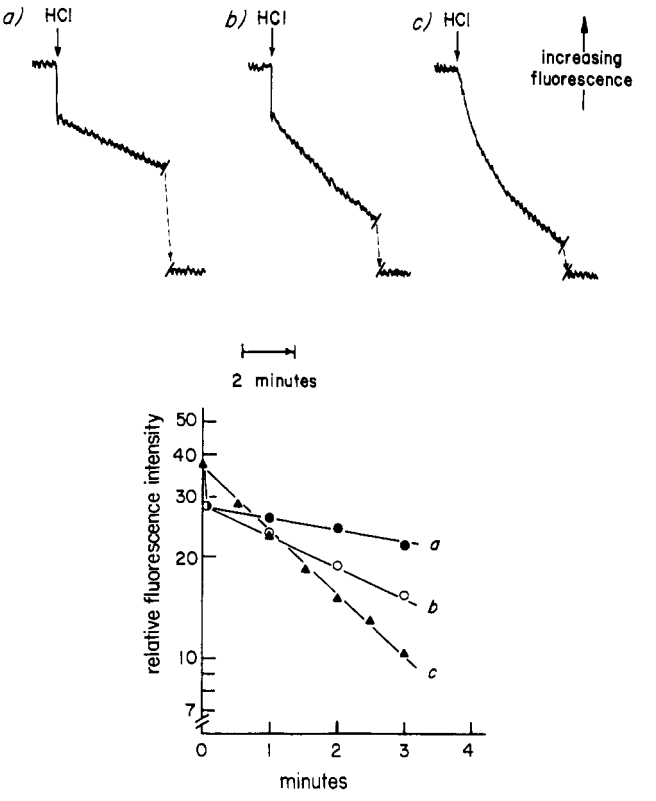

FIGURE 1: Effect of passing asolectin vesicles through mixing chamber and flow system of rapid-mixing instrument on kinetics of transmembrane proton influx. Pyranine-containing vesicles suspended in 0.1 M KCl, 5 mM Tricine/KOH, and 5 mM Mes/KOH (pH 8.2) at a concentration of 350 μg of phospholipid/mL were subjected to a rapid decrease in external pH (final pH 6.65) in a conventionally stirred fluorescence cuvette as described under Experimental Methods. Note the biphasic nature of the fluorescence decrease (trace a). Vesicles used in the experiment shown in trace b were pushed through the rapid-mixing flow system by manual operation of the sample drive syringes and collected from the stop syringe drainport before being assayed for proton influx. Vesicles used in the experiment shown in trace c were "fired" through the rapid-mixing flow system by using multiple bursts of 70 psi of N_2 to propel the drive syringes. The vesicle suspension was collected from the stop syringe and assayed for proton influx as described above. A semilogarithmic replot of the fluorescence changes is shown below the traces.

Results

Previous studies have shown that the fluorescence intensity of entrapped pyranine can be used as a reliable reporter of internal aqueous hydrogen ion concentration in unilamellar vesicles (Kano & Fendler, 1978; Clement & Gould, 1981a), with large changes in fluorescence intensity arising from changes in pH over the range pH 6.5–8.5. When the external pH of a stirred suspension of asolectin vesicles preequilibrated at pH 8.2 is rapidly lowered, a biphasic change in the intravesicular pH is observed (Figure 1). This biphasic pattern has been postulated to result from an initial very rapid electrogenic proton influx, followed by a much slower, counterion-limited electroneutral proton–counterion exchange. Support for this explanation comes from a variety of observations: (a) there is essentially no binding of pyranine to the external surface of anionic vesicles, (b) increasing vesicle permeability selectively to charge-compensating counterions accelerates the slow, secondary portion of the fluorescence response, and (c) the proportion of the total fluorescence change occurring

Table I: Effect of Phosphatidic Acid on Pyranine Fluorescence Changes in DMPC Vesicles[a]

phosphatidic acid (%)	ΔF^{fast} [b] (%)	pH_{in} [c]	$\Delta[H_{in}]$ [d] (M)
0	67.9 ± 5.8	7.21 ± 0.05	5.51×10^{-8}
5	58.9 ± 2.0	7.29 ± 0.02	4.47×10^{-8}
10	47.5 ± 4.6	7.39 ± 0.04	3.41×10^{-8}

[a] Unilamellar DMPC vesicles containing the indicated amount of phosphatidic acid were prepared as described under Experimental Methods. Vesicles which had been preequilibrated at pH 8.2 were subjected to a rapid decrease in external pH (final pH 6.65). Reaction conditions were essentially as described in the legend to Figure 1a. [b] Percent of the total change in the fluorescence of vesicle-entrapped pyranine occurring during the initial, rapid portion of the fluorescence decrease after acidification of the external medium. The standard deviations were determined from four identical experiments. Since, during their formation, zwitterionic DMPC vesicles bind a small amount of pyranine on their external surface (Clement & Gould, 1981a), it was necessary to correct for that portion of the fluorescence change (14%) arising from the externally bound probe. DMPC vesicles incorporating ≥5% phosphatidic acid did not bind pyranine. External pyranine binding was determined as described elsewhere (Clement & Gould, 1981a). [c] Internal pH at the end of the fast fluorescence decrease, calculated from the relative pyranine fluorescence intensity as described elsewhere (Clement & Gould, 1981a). [d] Calculated from the initial internal pH and the pH at the end of the fast component of the pyranine fluorescence change.

during the initial fast component is variable, becoming much smaller when the pH transition takes place at more acidic values, where a greater number of protons must cross the vesicle membrane during pH equilibration (Clement & Gould, 1981a).

This interpretation for the biphasic pyranine fluorescence response is very important since it implies that asolectin vesicle bilayers have a rather high intrinsic permeability to protons and are much less permeable to other monovalent ions. For this reason, a number of experiments were performed to further test this model. Essentially identical biphasic fluorescence responses were obtained if, in place of HCl, the pH of the external medium pH was lowered by the addition of a small volume of a strong buffer solution preadjusted to the desired final external pH, eliminating the possibility that the rapid initial fluorescence decreases was somehow related to a transient very low local pH which could occur between the time the HCl is added and mixing is completed (mixing time ≅ 1 s). The volume in which the HCl or strong buffer solution was added to the vesicle suspension also did not affect the characteristic biphasic fluorescence pattern, after appropriate corrections for sample dilution were made. Biphasic fluorescence changes were also obtained from vesicles prepared and suspended in a chloride-free medium and pulsed with H_2SO_4, eliminating the possibility that the observed responses were related to transmembrane movement by the neutral HCl species (Clement & Gould, 1981b).

Finally, if the initial, fast fluorescence decrease is the result of very rapid, electrogenic proton influx, then the proportion of the total fluorescence change occurring during the fast phase should depend upon the *number* of electrically uncompensated protons crossing the vesicle bilayer, rather than the imposed pH gradient. That this is in fact the case is shown by the experiment presented in Table I, in which the internal buffering capacity of unilamellar DMPC vesicles was varied by including different amounts of phosphatidic acid in the vesicle bilayer. The percent of the total fluorescence change associated with the fast kinetic component following a pH 8.2 → 6.65 transition was found to decrease with increasing proportions of phosphatidic acid in the membrane, so that the

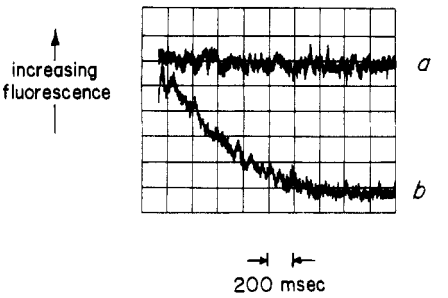

FIGURE 2: Changes in fluorescence intensity of vesicle-entrapped pyranine following an abrupt decrease in external pH utilizing a rapid-mixing technique. Pyranine-containing unilamellar asolectin vesicles (650 μg of phospholipid/mL) suspended in 0.1 M KCl, 5 mM Tricine/KOH, and 5 mM Mes/KOH (pH 8.2) were rapidly mixed in a Durrum D-110 stopped-flow spectrofluorometer with an equal volume of (trace a) the same medium (pH 8.2) (minus vesicles) or (trace b) the same medium (minus vesicles) containing HCl sufficient to yield a final pH, after mixing, of 6.65. Valinomycin (125 μM) was added to the vesicle suspension several minutes prior to mixing in order to eliminate rate-limiting counterion fluxes.

internal vesicle pH, at the end of the fast phase, was significantly more alkaline in vesicles containing the additional phosphatidic acid buffering residues. This result is entirely consistent with the conclusion that the initial fast kinetic component of the pyranine fluorescence change results from rapid, electrogenic proton movement into the vesicles.

In an attempt to resolve the kinetics of the initial fast fluorescence change, we utilized a rapid-mixing spectrofluorometer to mix a suspension of pyranine-containing asolectin vesicles (preincubated at pH 8.2) with an equal volume of mildly acidic buffer (final pH after mixing was 6.65). Preliminary experiments indicated that the resulting fluorescence decrease was monophasic, with an apparent half-time[3] of several minutes. Furthermore, the entire fluorescence decrease observed was accelerated by valinomycin (see below) or the permeant anion SCN^- (not shown), indicating that proton influx was limited by charge-compensating counterion redistributions. One possible explanation for the absence of the characteristic biphasic fluorescence response in the rapid-mixing experiments is that the initial rapid fluorescence decrease was occurring during the ~1 ms in which the freshly mixed vesicle–buffer solution travels from the mixing chamber to the observation cuvette. This does not seem likely, however, since the same initial fluorescence level after mixing was observed when vesicles were mixed with acidic buffer (final pH 6.65) or with alkaline buffer [final pH (=initial pH) 8.2] (Figure 2). Thus, the influx of protons into asolectin vesicles following their rapid dilution into acidic medium in the rapid-mixing apparatus appears to be genuinely monophasic.

It was also noted in preliminary experiments that the rate of the monophasic fluorescence decrease observed in the rapid-mixing experiments was always several times faster than the corresponding rate of the valinomycin-sensitive portion of the biphasic fluorescence decrease observed in conventional mixing experiments, suggesting that the rapid-mixing apparatus itself was somehow modifying the properties of the vesicle suspension. This possibility was further investigated by per-

[3] In order to quantify the effects of ionophores on the rate of H^+ diffusion into the vesicles, the half-time for the fluorescence change was determined from oscilloscope traces of the fluorescence decrease. It should be emphasized that, because the fluorescence intensity of pyranine is not linearly proportional to hydrogen ion concentration over the pH range investigated [see Clement & Gould (1981a)], the half-time for the *fluorescence* decrease actually corresponds to the point in the H^+ influx reaction at which the internal aqueous pH equals 7.43.

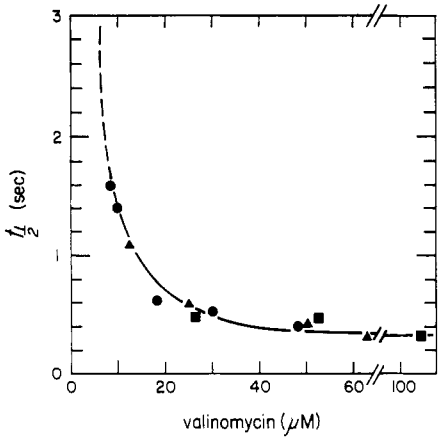

FIGURE 3: Effect of valinomycin concentration on rate of transmembrane H^+ equilibration following a rapid change in external pH. Reaction conditions were as described for Figure 2, trace b, except that the valinomycin concentration was varied. The concentrations given are the final concentrations, after mixing. The values for $t_{1/2}$ represent the time required for 50% of the pyranine fluorescence decrease to occur and correspond to the point at which the internal pH is 7.43. The different symbols represent data from three separate vesicle preparations. The $t_{1/2}$ observed in the absence of valinomycin was 130 s.

forming an experiment in which a suspension of asolectin vesicles was pushed through the stopped-flow mixing system (a) manually, when the sample drive syringes were operated slowly by hand, and (b) automatically (as in normal rapid-mixing experiments), when the sample syringes are driven rapidly by bursts of high-pressure nitrogen (70 psi). In each case the vesicle suspension was mixed with an equal volume of identical buffer (pH 8.2, containing no vesicles), and the resulting solution was collected from the instrument stop syringe. Aliquots (2 mL) of the vesicle suspensions were then pulsed with a small volume of HCl (sufficient to lower the external pH from 8.2 to 6.65) in a conventionally stirred fluorescence cuvette as described under Experimental Methods, and the change in fluorescence intensity of entrapped pyranine for each vesicle suspension was recorded (Figure 1). Vesicles which had been manually pushed through the stopped-flow mixing system, while still exhibiting a biphasic fluorescence decrease, exhibited an accelerated secondary (slow) kinetic component when compared to vesicles not exposed to the rapid-mixing flow system, suggesting that counterion permeability had been slightly enhanced by the treatment. Vesicles which had been forced through the stopped-flow apparatus under the conditions normally used for making rapid kinetic measurements not only exhibited an increase in the rate of counterion-limited proton influx but also exhibited the characteristic *monophasic* fluorescence decrease seen in similar experiments performed in the rapid-mixing instrument (cf. Figure 2).

At the moment, no explanation for these observations is available, although it is conceivable that (a) forcing the vesicles through the small-bore conduits within the rapid-mixing system and/or (b) the large, transient hydraulic pressure generated on the vesicle suspension at the abrupt termination of sample flow through the mixing system leads to structural or other changes in the vesicles themselves or to a change in the makeup of a heterogeneous vesicle population, resulting in the observed effects. It is clear from these experiments, however, that there is little or no vesicle breakage during the rapid-mixing procedure, since any such breakage would have released the entrapped pyranine into the external medium, where it would respond instantaneously to any change in external pH (cf. Figure 1c).

Table II: Effect of Bis(hexafluoroacetonyl)acetone (1799) on H^+ Equilibration across Unilamellar Asolectin Vesicle Membranes[a]

1799 (mg/mL)	$t_{1/2}$[b] (ms)
0.0	310
0.09	49
0.14	9
0.50	<1

[a] Reaction conditions were essentially as described in the legend to Figure 2 (trace b). 1799 was added to the vesicle suspension several minutes prior to mixing in the stopped-flow apparatus. [b] Time required for the fluorescence of vesicle-entrapped pyranine to decrease by 50% (corresponding to an internal pH change from 8.2 to 7.43).

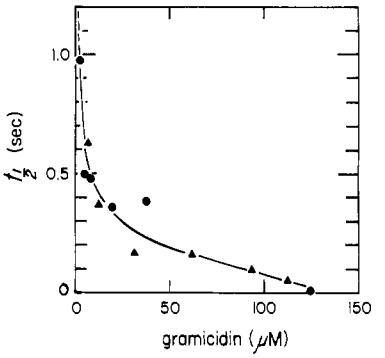

FIGURE 4: Effect of gramicidin concentration on rate of transmembrane H^+ equilibration following a rapid change in external pH. Reaction conditions were essentially as described in the legend to Figure 3, except that valinomycin was omitted and gramicidin was added as indicated. The concentrations of gramicidin given are the final concentrations, after mixing. The different symbols represent data from two separate vesicle preparations. The $t_{1/2}$ observed in the absence of gramicidin was 145 s.

Regardless of the explanation for these phenomena, however, it is clear that the rate-determining process for the monophasic fluorescence decrease observed after an 8.2 → 6.65 pH shift in the rapid-mixing system is the electrically driven redistribution of charge-compensating counterions. In the presence of valinomycin the half-time for the fluorescence decrease could be lowered to a plateau of about 300–400 ms, but not further (Figure 3). Under these conditions, the observed half-time for the proton influx reaction must therefore be determined by the intrinsic membrane permeability to hydrogen ions. This conclusion is supported by the observation that, in the presence of excess valinomycin, the rate of transmembrane proton equilibration can be further increased by the proton ionophore bis(hexafluoroacetonyl)acetone (1799) (Table II).

In lipid bilayers the ionophoretic antibiotic gramicidin forms dimeric, transmembrane channels which are capable of conducting a variety of monovalent cations, including protons, at very high flux rates (Goodall, 1970; Urry et al., 1971; Rudin, 1967; Hladky and Haydon, 1970). Incorporation of gramicidin channels into asolectin vesicles prior to the abrupt change in external pH resulted in a dramatic acceleration of the rate of transmembrane proton equilibration, with half-times approaching the time resolution of the mixing system (1 ms), indicating that the hydrogen ion permeability barrier, which exists even in the presence of excess valinomycin, is absent in gramicidin-containing vesicles (Figure 4).

Discussion

Previous studies of hydrogen ion diffusion across natural (Mitchell & Moyle, 1967; Crandell et al., 1971) and artificial (Scarpa & DeGier, 1971; Nichols & Deamer, 1980) phos-

pholipid bilayer membranes relied upon measurements of small changes in the hydrogen ion concentration of a weakly buffered suspending medium or upon the pH-dependent distribution of membrane-permeant amine dyes between the internal and external aqueous compartments (Nichols et al., 1980). The studies we have reported here and elsewhere (Clement & Gould, 1981a,b) are based upon a completely different approach, in which membrane-impermeant, pH-sensitive fluorescent molecules are entrapped within the vesicle's internal aqueous compartment. A number of important advantages are offered by this approach, including high sensitivity and time resolution and convenient calibration. However, measurements of H^+ movements into or out of vesicles by the entrapped probe technique are only reliable under certain conditions. It is important, for example, that the probe molecule be sufficiently hydrophilic so that it cannot readily cross the vesicle membrane and that the probe molecule remain free in solution without binding to either the internal or external surface of the bilayer. When vesicles having a net negative surface charge (e.g., asolectin) are employed, the pyranine polyanion has been shown to meet each of these requirements, so that fluorescence changes of vesicle-entrapped pyranine can be utilized as a convenient indicator of changes in the vesicle's internal aqueous compartment (Kano & Fendler, 1978; Clement & Gould, 1980, 1981a).

The unexpectedly high proton conductances we have measured for asolectin vesicle membranes could conceivably be explained by the presence of a highly active proton ionophore contaminating the phospholipid preparation. Such an explanation, however, cannot be applied to vesicle membranes formed from pure synthetic lipids, which also exhibit a very high proton conductance. Nor does it seem likely that the pyranine is increasing proton conductance by acting as an ionophore or somehow modifying the bilayer structure, since identical results were obtained when the probe/vesicle ratio was varied from 0.5 to 4 (assuming $\sim 3 \times 10^3$ phospholipid molecules/vesicle; Watts et al., 1978).

On the other hand, it is clear that the rapid-mixing procedure employed for the experiments reported here is responsible for some as yet poorly understood changes in the vesicle suspension. Vesicles which have been subjected to the transient hydraulic forces generated by the rapid-mixing apparatus, along with the shearing forces encountered during turbulent flow through the small-bore flow passages, exhibit an increased permeability to one or both of the counterions present in the suspending buffer (K^+, Cl^-) and may also exhibit modified proton conductance characteristics when the rate limitation imposed by these counterion fluxes is removed by valinomycin. The extent to which this latter phenomenon is occurring cannot be assessed from the data presented here. However, it is important to note that the rate of H^+ influx observed in the rapid-mixing experiments, when valinomycin is present ($t_{1/2} \cong 300$ ms), is consistent with the rate of H^+ influx observed in analogous, conventional mixing experiments ($t_{1/2} < 1$ s; Clement & Gould, 1981a,b). It therefore seems reasonable to conclude that the natural permeability of phospholipid bilayer vesicles to hydrogen ions is surprisingly high and much greater than their permeability to other small monovalent ions.

Phospholipid vesicles formed by sonication (Racker, 1973), detergent dilution (Racker et al., 1975), or detergent dialysis (Kagawa & Racker, 1971) have been widely utilized for the reconstitution of an impressive array of membrane-dependent enzymatic systems, including the mitochondrial and chloroplast ATP synthase complexes (Kagawa & Racker, 1971; Carmelli & Racker, 1973; Racker, 1973; Winget et al., 1977), the three "sites" of oxidative phosphorylation (Ragan & Racker, 1973; Leung & Hinkle, 1975; Racker & Kandrach, 1971), the adenine nucleotide transporter (Shertzer & Racker, 1974), the bacteriorhodopsin proton pump (Racker & Stoeckhenius, 1974; Racker, 1973), the sarcoplasmic reticulum Ca^{2+} pump (Knowles & Racker, 1975), the chromaffin granule catecholamine carrier (Maron et al., 1979), and the synaptosomal γ-aminobutyrate and L-glutamate carriers (Kanner, 1978; Kanner & Sharon, 1978), among others. The phospholipid mixture used in each of the reconstitutions cited above was asolectin, which in many cases yielded better reconstituted activities than pure synthetic or natural phospholipids either individually or in defined mixtures (Kagawa et al., 1973; Leung & Hinkle, 1975; Winget et al., 1977). Indeed, the reconstituted activities of the bacteriorhodopsin proton pump and the mitochondrial ATP synthase complex were actually higher in asolectin membranes than in membranes formed from *Halobacterium* or mitochondrial phospholipids, respectively (Racker, 1973; Kagawa et al., 1973).

Because a large number of the enzymatic activities which have been successfully reconstituted into asolectin vesicles utilize transmembrane hydrogen ion gradients as energy-rich intermediate states (Mitchell, 1968), the rather high permeability of asolectin bilayers to hydrogen ions is somewhat disquieting. Furthermore, this high intrinsic permeability to hydrogen ions may be a general property of vesicular phospholipid bilayers, since results similar to those reported here have also been observed in egg phosphatidylcholine (Nichols & Deamer, 1980; Clement & Gould, 1981a) and synthetic DMPC, DPPC, and DLPC vesicles (N. R. Clement and J. M. Gould, unpublished experiments), in vesicles formed by a variety of procedures, and in large multilamellar liposomes (Clement & Gould, 1981a).

The mechanism by which protons are apparently able to cross phospholipid bilayers so readily is not at all clear, although it is obvious from a number of studies (Clement & Gould, 1981a,b; Papahadjopoulos et al., 1972; Hauser et al., 1973; Nichols et al., 1980; Nichols & Deamer, 1980) that this mechanism either is unavailable to other monovalent cations or conducts them with greatly reduced efficiency. This observation has led Nichols & Deamer (1980) to postulate that protons can cross lipid bilayers via a hydrogen-bond exchange mechanism, similar to the mechanism of proton migration in liquid water and ice (Eigen & DeMaeyer, 1958), utilizing H_2O molecules dissolved in the membrane hydrocarbon.

Based upon the measured solubility of H_2O in *n*-alkanes (Schatzberg, 1963, 1965) and *n*-alkenes (Black et al., 1948), it is possible to calculate[4] that, even in the very small vesicles utilized in this study, at least 30–40 H_2O molecules would be dissolved in the hydrocarbon interior of each vesicle membrane. It would appear, therefore, that sufficient intrahydrocarbon H_2O could exist to form transmembrane, hydrogen-bonded H_2O chains linking the bound (unstirred) H_2O layers on either side of the bilayer.

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[4] These calculations are based upon an average vesicle = 3×10^3 phospholipid molecules with a membrane volume of 0.94 mL/g of phospholipid (Watts et al., 1978) and an average H_2O solubility in the membrane hydrocarbon of $\sim 1.3 \times 10^{-2}$ mol of H_2O/mol of phospholipid. The latter value was estimated by multiplying the solubility of H_2O in tetradecane and hexadecane (1.2×10^{-3}–1.3×10^{-3} mol/mol of hydrocarbon; Schatzberg, 1963) by the average increase in hydrocarbon H_2O solubility arising from the presence of unsaturation in the hydrocarbon (8–9-fold; Black et al., 1948).

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