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Net proton-hydroxyl permeability of large unilamellar liposomes measured by an acid-base titration technique

(pH gradients/ether-injected liposomes/valinomycin)

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ABSTRACT **The net proton-hydroxyl permeability of large unilamellar liposomes has been measured by an acid-base pulse titration technique and has been determined to be several orders of magnitude greater than that measured for other monovalent ions. This permeability is relatively insensitive to variations in lipid composition. Proton permeability and hydroxyl permeability vary with pH 6 to 8, and this variation can occur in the absence of alterations in surface charge density resulting from titrations of acidic and basic groups on the lipids. In order to account for the exceptionally high proton-hydroxyl permeability with respect to other monovalent ions, we propose that protons or hydroxyls or both interact with clusters of hydrogen-bonded water molecules in the lipid bilayer, such that they are transferred across the bilayer by rearrangement of hydrogen bonds in a manner similar to their transport in water and ice.**

The regulation of proton and hydroxyl flux across biological membranes is central to cell function. For example, proton and hydroxyl activities across many cell membranes are not in equilibrium with the electrochemical potential, suggesting that active transport of these ions is involved in the regulation of intracellular pH (1). Because a number of intracellular metabolic processes are affected by small changes in pH (2), several investigators have suggested that the control of cell division and growth is regulated by shifts in intracellular pH (3–6). In addition, proton and hydroxyl transport across organelle membranes is a primary energy source driving ATP synthesis (7–9), and maintenance of acidic compartments is essential for proteolytic enzyme activity in lysosomes (10) and for catecholamine uptake and storage in secretory vesicles (11, 12).

It follows from these considerations that knowledge of passive proton-hydroxyl flux is essential to understanding mechanisms by which proton gradients are maintained across membranes. Because the lipid bilayer moiety of membranes represents the major barrier to ionic permeation, proton-hydroxyl permeability of lipid bilayers should be determined in order to provide a standard for comparison with biological membranes. For this reason, in past studies we have investigated proton-hydroxyl flux by monitoring the decay of relatively large buffered pH gradients (2–3 pH units) with a fluorescent probe technique (13). In the present study we have directly measured the decay of small pH gradients (0.1–0.2 pH unit) with a glass electrode method, and the results confirm and extend our earlier findings. A relationship expressing net proton-hydroxyl permeability could be developed from the flux measurements, and we propose a mechanism to explain the remarkably high measured proton-hydroxyl permeability.

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METHODS

Liposome Preparation. Liposomes were prepared by injecting 4 ml of diethyl ether containing 2 mM lipid into 4 ml of a highly buffered (50 mM zwitterionic buffer) salt solution warmed to 60°C (14, 15). The standard mixture was egg phosphatidylcholine with 10 mol % phosphatidic acid. Phospholipids were purchased from Avanti Biochemicals (Birmingham, AL) and cholesterol was purchased from Sigma. The liposomes were filtered through a 0.45-μm Millipore filter to remove larger lipid structures and then through Sephadex G-50 (1 × 30 cm column) into a lightly buffered (0.5 mM zwitterionic buffer) isoosmotic solution. The osmolarity of the external solution was adjusted with sucrose in order that the concentration of salt was the same inside and out. The Sephadex beads were routinely removed from the column, washed in methanol and water, and repacked for each preparation.

Measurement of Proton-Hydroxyl Flux. The external pH was monitored by a Radiometer 26 pH meter attached to a Linear Instruments (Irvine, CA) model 251 chart recorder. A 6-mm glass electrode was inserted into a small sample vial containing 2 ml of the liposome solution. Argon was continuously bubbled through the liposome solution to remove carbon dioxide. After the external pH stabilized following the removal of the carbon dioxide, small pH gradients were established across the liposome membranes by injecting acid or base. Each gradient was allowed to equilibrate until the external pH was constant, at which time the external and internal pH values were equal. This procedure could be repeated several times for each preparation. From the ratio of the change in internal pH to the change in external pH ($\delta pH_i/\delta pH_o$) after the return to equilibrium, we could predict the internal pH from the corresponding external pH. All measurements were performed at room temperature, 23°C.

Calculation of Net Proton-Hydroxyl Permeability. The net flux of proton and hydroxyl ions across a membrane (J_{net}) is the summation of the unidirectional fluxes of protons and hydroxyls in and out. If electrical forces are ignored, the following equation describes the flux as dependent on the activities of protons and hydroxyls inside and outside the vesicles:

$$J_{net} = P_H([H^+]_o - ([H^+]_i) + P_{OH}([OH^-]_i - [OH^-]_o). \quad [1]$$

P_H and P_{OH} are the permeability coefficients for protons and hydroxyls, respectively; $[H^+]$ and $[OH^-]$ are the proton and hydroxyl activities; and the subscripts "o" and "i" refer to outside and inside the vesicles, respectively. This flux equation is based on the assumptions that (*i*) the flux is not limited by the development of a diffusion potential, (*ii*) the proton and hy-

Abbreviation: CTAB, cetyltrimethylammonium bromide.

droxyl permeabilities are independent of the direction of flux, and (*iii*) the proton and hydroxyl ions flux independently. The validity of these assumptions will be discussed later.

For a small acid or base pulse around pH 7.0, the difference in proton concentrations across the membranes is approximately equal and of opposite sign to the difference in hydroxyl concentrations.

$$[H^+]_o - [H^+]_i = [OH^-]_i - [OH^-]_o. \quad [2]$$

If we define P_{net} such that

$$P_{net} = P_{OH} + P_H \quad [3]$$

then

$$J_{net} = P_{net}([H^+]_o - [H^+]_i). \quad [4]$$

The net proton-hydroxyl flux can be calculated from the derivative of the external pH with respect to time (dpH_o/dt) multiplied by the appropriate factors.

$$J_{net} = \frac{dpH_o}{dt}\frac{B_oV_o}{A}. \quad [5]$$

B_o is the buffer capacity of the external solution; V_o is the external volume; and A is the total surface area of the vesicles. Buffers were selected such that the small pH range used for each experiment would be near their pK, resulting in a constant value for B_o. B_o was determined by measuring the initial external pH shift after the addition of a known quantity of acid or base. The surface area was estimated by assaying the amount of phospholipid (16) and assuming a packing area of 55 Å^2 per molecule phospholipid for pure phospholipid vesicles (17) and 50 Å^2 for vesicles containing a mixture of phospholipid and cholesterol (18). The molecular area of cholesterol is constant and was taken as 38 Å^2 (19).

After a given pH gradient has returned to equilibrium, pH_i equals pH_o. Because the initial internal pH (pH_i^i) and external pH (pH_o^i) are known, the ratio of the change in internal pH to the change in external pH can be calculated ($\delta pH_o/\delta pH_i$). This ratio is constant during the decay process and can be used to predict the internal pH for each corresponding value for pH_o as shown in the following equation:

$$pH_i = R(pH_o - pH_o^i) + pH_i^i \quad [6]$$

in which R is the ratio $\delta pH_i/\delta pH_o$. Because proton activity is, by definition, the antilog of the negative pH, Eqs. **4**, **5**, and **6** can be combined to give a theoretical prediction for the external pH decay:

$$\frac{dpH_o}{dt} = P_{net}\frac{A}{B_oV_o}(10^{-pH_o} - 10^{-R[(pH_o - pH_o^i) - pH_i^i]}) \quad [7]$$

This differential equation does not have a simple analytical solution. We therefore calculated a value for P_{net} by repeatedly substituting P_{net} into Eq. **7** and integrating numerically until a value was selected that accurately predicted the actual external pH recording at a given time.

RESULTS

The results of a typical experiment are shown in Fig. 1. The solid line represents the actual recording of the external pH, and the broken line indicates the internal pH predicted from Eq. **6**. Because the decay rates for a given magnitude pH pulse are essentially the same regardless of the direction of the pulse, several permeability measurements at the same pH could be made by alternating acid and base pulses. The external pH predicted from Eq. **7**, represented by the circles, accurately describes the measured external pH as the imposed gradients return to equilibrium.

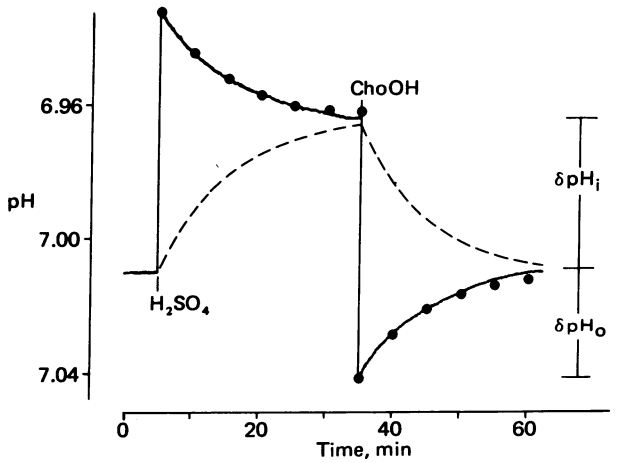

FIG. 1. Time course of the external pH during pulsed acid and base titrations: 0.5 μl of 0.05 M H_2SO_4 or 0.1 M choline hydroxide (ChoOH) was added as indicated, and the external pH was recorded (—). The internal pH of the vesicles (- -) was predicted from Eq. **6**. Theoretical values for external pH (●) were calculated from Eq. **7**. Liposomes, prepared as described in the text, contained 50 mM *N*-(2-acetamido)-2-aminoethanesulfonic acid (pK 6.9), 75 mM KCl, and 25 mM choline (internal) or 0.5 mM *N*-(2-acetamido)-2-aminoethanesulfonic acid, 75 mM KCl, and 75 mM sucrose (external). δpH_i and δpH_o are the total change in internal and external pH, respectively, after the initial pH gradient has returned to equilibrium.

Elimination of Vesicle Rupture, Buffer Leakage, and Carbon Dioxide Equilibration as Sources of Proton and Hydroxyl Flux. Vesicle rupture and buffer leakage conceivably could contribute to the equilibration of the imposed pH gradients. To test this possibility, the external buffer capacity of the liposome solutions was monitored over long time intervals. The release of buffer from the liposomes by either mechanism would result in an increase in the external buffer capacity. No significant increase in the external buffer capacity was detected after allowing the liposome solutions to equilibrate for more than 24 hr. We concluded that neither vesicle rupture nor buffer leakage significantly increased the rate of proton-hydroxyl flux and the resultant permeability coefficient.

By continually flushing the liposome solutions with the inert gas argon, carbon dioxide was removed before the pH pulses were begun, eliminating the possibility of dissolved carbon dioxide and bicarbonate acting to equilibrate pH gradients.

There remains the possibility that ether-injection liposomes present a special case in regard to ion permeability, but this seems unlikely for several reasons. First, this preparation was chosen because of the primarily unilamellar character and high volume trapping efficiency of the liposomes. Highly sonicated liposomes would provide a more uniform preparation but do not contain enough buffer to permit measurements under our conditions. In one experiment, to test whether the ether-injection method markedly alters the lipid, liposomes were prepared by brief sonication (1 min) of the standard lipid mixture. This preparation was not homogeneous but did trap sufficient buffer to permit proton flux measurements by the 9-aminoacridine method (13). The calculated permeability was in the expected range.

Later in this paper we will describe calculations of permeability using conductance data obtained for mitochondrial membranes and will show that the proton permeability of mitochondria is greater than that of liposomes. Finally, even if ether-injection liposomes do present a special case for absolute measurements of ion permeability, there remains the observed relative difference in proton and sodium permeabilities. These

were measured in the same preparation and represent the main thrust of our report.

Counterion Flux Does Not Limit Proton-Hydroxyl Flux. Because the model proposed in Eq. 7 to describe proton-hydroxyl flux includes only the chemical driving forces and not electrical forces, we had to establish experimental conditions such that no proton-hydroxyl diffusion potential would develop upon pH gradient decay. If the intrinsic bilayer permeability allowed proton and hydroxyl flux to approach the same magnitude as the combined counterion flux, a significant diffusion potential would develop and retard the rate at which the pH gradients returned to equilibrium, resulting in an underestimate of the proton-hydroxyl permeability. If counterion flux were limiting proton-hydroxyl flux, the addition of valinomycin to a solution of liposomes containing potassium would result in an increase in the proton-hydroxyl flux as the diffusion potential was eliminated. Because valinomycin has been shown to be more selective for protons than for potassium (20), its effect on the potassium counterion permeability had to be distinguished from its direct effect on proton permeability.

The experiment illustrated in Fig. 2 was designed to distinguish between these two possibilities. Two solutions of liposomes were prepared. The first contained potassium and the second contained choline as the only cation. Because valinomycin does not increase choline permeability significantly, an increase in the apparent net proton-hydroxyl permeability in the choline solution would result from increased proton-hydroxyl permeability alone; an increase measured in the potassium solution would be a result of an increase in both the proton-hydroxyl and the potassium counterion permeability. Because the increase in the apparent permeability upon valinomycin addition actually was slightly greater for the choline solution than for the potassium solution, we concluded that the proton-hydroxyl flux was not limited by counterion flux and that the experimental conditions were consistent with the limits of the theoretical flux model. Presumably, the permeability of the liposomes to chloride is high enough so that the chloride current flux is sufficient to short-circuit the development of a proton-hydroxyl potential.

The fact that the apparent proton-hydroxyl permeability was slightly less in the potassium solution than in the choline solution for a given amount of valinomycin is consistent with the idea that potassium ions compete with protons for the valinomycin binding sites, resulting in an inhibition of the direct increase in proton permeability.

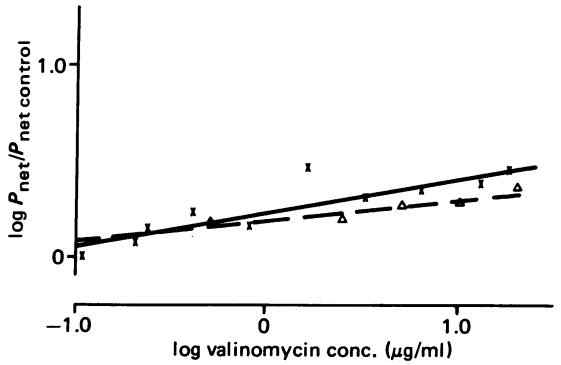

FIG. 2. Effect of valinomycin on net proton-hydroxyl permeability calculated for the same liposome preparations as the valinomycin concentration was increased in the presence of potassium (△) or choline (x). The liposome solutions contained 2.0 mM 9:1 phosphatidylcholine/phosphatidic acid mixture. The potassium solutions were prepared as described in Fig. 1. The choline solutions were as in Fig. 1 except that choline chloride was used in place of potassium chloride. The permeability calculated after valinomycin addition was standardized by dividing it by the permeability measured in the absence of valinomycin ($P_{net\ control}$). Valinomycin was dissolved in ethanol. Control additions of ethanol had no effect on net proton-hydroxyl permeability.

It is interesting to note that the net proton-hydroxyl permeability did not follow the first-power dependence on valinomycin concentration, as has been demonstrated for potassium (21). The first-power dependence is based on the assumption that the cation–valinomycin complex is the major charge-carrying species within the membrane, and this does not appear to be a valid assumption for protons. Although valinomycin has been shown to have a greater affinity for protons than for potassium (20), the intrinsic proton-hydroxyl permeability was so high that the proton–valinomycin complex carried only a fraction of the total proton-hydroxyl flux. As a result, proton-hydroxyl permeability would not be characterized by the first-power dependence on valinomycin concentration expected for potassium permeability.

Effect of Lipid Composition on Proton-Hydroxyl Permeability. The net proton-hydroxyl permeability coefficients measured for liposomes composed of several different lipid compositions are presented in Table 1. The highest permeability was found for liposomes prepared with phosphatidylcholine and cetyltrimethylammonium bromide (CTAB). CTAB is a positively charged quaternary ammonium radical attached to a 16-carbon alkyl chain. The addition of CTAB increases the permeability about 5-fold over that measured in liposomes containing 10% phosphatidic acid. The increased permeability probably results from the detergent effect of the CTAB. There seems to be no simple relationship between surface charge and permeability because phosphatidylserine liposomes also have a slightly higher permeability compared to those with phosphatidylcholine/phosphatidic acid mixture. Liposomes prepared with 50% cholesterol had the lowest permeability. Overall, the net proton-hydroxyl permeability appeared to be relatively insensitive to variations in lipid composition.

Effect of pH on Net Proton-Hydroxyl Flux. The small pH gradients used for these experiments permitted measurement of the proton-hydroxyl flux in solutions buffered at different pH values while the magnitude of the pH gradient was held constant. The initial net fluxes resulting from 0.1 pH unit-pulses centered around pH 6, 7, and 8 could be measured and compared to a system of theoretical curves predicted from Eq. 1. If the separate proton permeability (P_H) and hydroxyl permeability (P_{OH}) coefficients are constant over this pH range, the three measured fluxes would fall on one of the theoretical lines and the values for the separate coefficients could be calculated. The solid lines in Fig. 3 are examples chosen from the infinite set of possible lines relating the dependence of the net flux on pH. The magnitude of the permeability coefficients selected to generate these curves was arbitrarily chosen to provide a value for the net flux in the range of the experimental

Table 1. Effect of lipid composition on net proton-hydroxyl permeability

Lipid	Mole ratio	% charge	P_{net}, cm × 10^4/sec (mean ± SD)
Chol/PtdCho/PtdA	10:9:1	10 (−)	3.2 ± 1.1 (14)
PtdCho/PtdA	9:1	10 (−)	4.3 ± 2.1 (11)
PtdSer		100 (−)	6.5 ± 1.9 (12)
PtdCho/CTAB	9:1	10 (+)	20.8 ± 13.9 (24)

Abbreviations: Chol, cholesterol; PtdCho, phosphatidylcholine; PtdA, phosphatidic acid; PtdSer, phosphatidylserine; CTAB, cetyltrimethylammonium bromide. Number of measurements is given in parentheses.

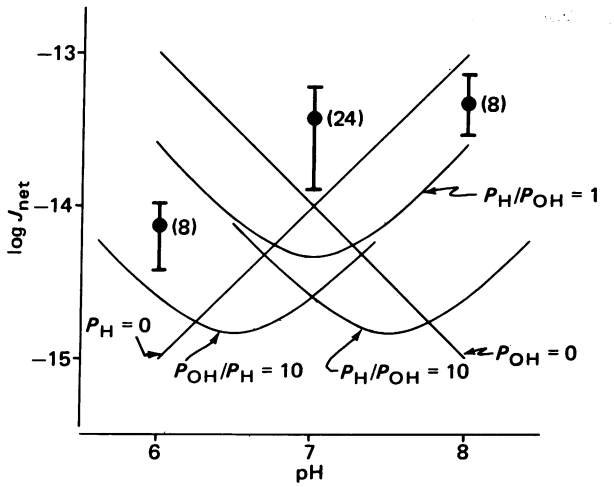

FIG. 3. pH dependence of net proton-hydroxyl flux. The lines represent a few of the infinite possibilities for the dependence of the net proton-hydroxyl flux on pH as predicted from Eq. 1 by assuming that the proton permeability and the hydroxyl permeability remain constant. The absolute values for these coefficients were arbitrarily chosen such that the predicted minima would be in the same range as the measured fluxes. The mean (±SD; n in parentheses) measured net proton-hydroxyl flux (●) was calculated from Eq. 5. dpH_o/dt was taken as the initial slope of the pH recording after an acid or base pulse of constant magnitude (approximately 0.06 pH unit) at each pH. The liposome solutions contained 2.0 mM 9:1 phosphatidylcholine/CTAB mixture. The internal and external solutions were identical to those described in Fig. 1 for the experiments performed at pH 7. For the experiments performed at pH 6 and 8, 2-(N-morpholine)ethanesulfonic acid (pK 6.15) and N-tris(hydroxymethyl)methylglycine (pK 8.15), respectively, were substituted, in the same concentrations, for the buffer.

values. If either P_H or P_{OH} equals zero, the logarithm of the net flux would be linearly related to pH with a slope of +1 or −1, respectively. If P_H and P_{OH} are equal in magnitude, the expected curve is a parabola with a minimum at pH 7. The pH of the minimum shifts depending on the ratio of P_H to P_{OH}, as shown. The experimental measurements of the fluxes are shown by the black dots (± SD). These three points do not satisfy any of the possible theoretical curves, and we concluded that the permeability coefficients P_H and P_{OH} are not independent of pH. One or both of these coefficients must change as a function of pH in order to account for the measured fluxes.

The liposomes for these experiments were prepared from a 9:1 phosphatidylcholine/CTAB mixture. This lipid composition was selected in order to minimize changes in surface charge resulting from titration of ionizable groups as a function of pH. Pure phosphatidylcholine vesicles of this size form aggregates and are not suitable for these measurements. Phosphatidylcholine is isoelectric between pH 3 and 10 (22), whereas CTAB has no titratable charges. Therefore, the pH-dependent changes in the proton or hydroxyl permeability do not result from changes in the surface charge density.

DISCUSSION

Comparison with Previous Measurements of Proton-Hydroxyl Permeability. Nichols and Miller (23) and Kornberg *et al.* (24) have suggested that the high permeability measured for chloride ions in liposome membranes is a result of neutral HCl permeation. Kornberg *et al.* found that the logarithm of the measured chloride flux decreased linearly as the pH of the incubating solution increased. Although the absolute value of the slope of the relationship was much less than the expected slope of 1, they proposed that chloride crosses the lipid bilayer combined with protons. Our results (Fig. 3) do not confirm the linear dependence of logarithm of flux on pH expected if only HCl were permeating the membranes. In addition, we have measured similar proton-hydroxyl flux across liposomes in the absence of chloride or other monovalent anions. We conclude that proton-hydroxyl flux is not significantly affected by HCl permeation.

We previously measured the net proton-hydroxyl permeability of liposomes prepared by ether injection by an entirely different technique using 9-aminoacridine as a pH probe (13). The results of these measurements are in agreement with the measurements presented in this paper to within a factor of 4. The measurements are surprisingly similar, considering the differences in the technique. The 9-aminoacridine technique involves measuring the rate of change in the vesicle internal pH as a large pH gradient (approximately 3 pH units) decays to equilibrium. The average net proton-hydroxyl permeability was 1.44×10^{-4} cm/sec for liposomes prepared from 98% phosphatidylcholine and 2% phosphatidic acid. The measurements made by the technique reported in this paper measure the equilibration rates of very small pH gradients (approximately 0.1 pH unit). Our finding of similar proton-hydroxyl permeabilities by two independent methods lends confidence to the accuracy of these measurements.

Comparison of the Proton-Hydroxyl Permeability in Liposomes to That in Biological Membranes. It is interesting to compare these results with other measurements of proton-hydroxyl permeability in biological membranes. For example, Mitchell and Moyle (25) developed a pH pulse technique similar to that used in this paper to measure the net proton conductance of mitochondrial membranes. Although the mitochondrial membrane has a very low proton-hydroxyl conductance, 0.45 μmho/cm^2 at pH 7, the net proton-hydroxyl permeability calculated from the same data is relatively high, 1.34×10^{-3} cm/sec. Conductance depends not only on the ionic permeability but also on the number and distribution of ions available on either side of the membrane. Because the number of protons and hydroxyls at pH 7 is very low compared to physiological concentrations of sodium, potassium, and chloride ions, a low proton-hydroxyl conductance can reflect a very large permeability coefficient. The proton-hydroxyl permeability of mitochondrial membranes is in the same range as that found for pure phospholipid vesicles in this report and suggests that the intrinsic permeability of the phospholipid portion of the mitochondrial membrane can account for the passive proton-hydroxyl flux. Although the proton-hydroxyl permeability of these lipid vesicles is high compared to other ions, the vesicles provide a significant barrier to the free diffusion of protons and hydroxyls on a biological time scale. Similar conclusions were reached by Maloney (26), who used acid-base pH pulses to study conductance of bacterial membranes.

Proposed Mechanism for Proton-Hydroxyl Flux. The net proton-hydroxyl permeability is at least several orders of magnitude greater than the permeability of other monovalent cations and anions. We have found sodium permeability to be on the order of 10^{-10} cm/sec in ether-injected liposomes (13). Other studies of cation and anion permeabilities have used either multilamellar or sonicated liposomes and found permeabilities in the range of 10^{-13} to 10^{-14} cm/sec for monovalent cations and 10^{-11} to 10^{-12} cm/sec for chloride (27–30). It follows that proton-hydroxyl permeation must result from a unique mechanism not available to other cations and anions.

We propose that proton and hydroxyl ions are able to utilize associated water molecules in the hydrophobic portion of the bilayer. This interaction would permit transport of proton equivalents across lipid bilayers in a manner analogous to proton conductance in water and ice. The mobility of protons in water

is about 5–7 times greater than that of other cations as a result of their ability to be transferred along a series of hydrogen-bonded water molecules by rearrangement of the hydrogen bonds. Proton mobility is 50 times greater in ice than in water due to the optimal orientation and spacing of the water molecules for hydrogen bond exchange (31). Nagle and Morowitz (32) proposed a similar mechanism in which protons cross membranes along continuous chains of hydrogen bonds ("proton wires") formed from the side groups of transmembrane proteins.

Chains of associated water molecules within the bilayer would permit transport of protons and hydroxyls via hydrogen bond exchange. Although the amount of water contained within phospholipid bilayers and the degree to which it is associated have not been determined, several lines of evidence support the suggestion that associated molecules of water do exist in these bilayers. There is no doubt that water is present in the hydrophobic portion of the bilayer because lipid membranes are relatively permeable to water, with permeability coefficients on the order of 10^{-3} cm/sec (33–35). The measured solubility of water in bulk hydrocarbons is in the millimolar range for *n*-alkanes (36) and typically increases 5–8 times for an alkene of the same carbon chain length (37). Nuclear magnetic resonance studies have shown that at least the first 7 carbons in micelles composed of a 16-carbon detergent are in an aqueous environment (38). Finally, infrared spectroscopy has been used to show that there is a slight preference for water–water hydrogen bonds over water–alcohol bonding when small amounts of water are dissolved in octanol (39). In light of this evidence, it is reasonable to assume that a significant amount of water exists within the bilayer in the form of hydrogen-bonded strands which can facilitate proton and hydroxyl transport by the rearrangement of hydrogen bonds, as in water and ice. This proposed model accounts for the exceptionally high permeability of protons and hydroxyls compared to other ions and can serve to direct future investigations into the mechanism for their transport across phospholipid bilayers.

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