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Proton Flux Mechanisms in Model and Biological Membranes

David W. Deamer and J. Wylie Nichols
Department of Zoology, University of California, Davis, California 95616, and Department of Physiology, Emory University School of Medicine, Atlanta, Georgia 30322

Introduction

With the realization that electrochemical proton gradients play a fundamental role in cellular bioenergetics, new tools have been developed to monitor proton transport, membrane potentials and transmembrane pH gradients. This has led to a proliferation of knowledge about how protons interact with cell membranes. Besides the central function of the F_0F_1 ATPase in coupling proton transport to ATP synthesis, it has been found that E_1E_2 ATPases actively transport protons in membranes as diverse as lysosomes (Mego, 1975; Schneider, 1983), chromaffin granules (*reviewed by* Johnson, 1987), synaptic vesicles (Toll & Howard, 1978), clathrin-coated vesicles (Stone, Xie & Racker, 1983), gastric mucosal membranes (Forte & Wolosin, 1987; Sachs, 1987) and plasma membranes (Roos & Boron, 1982). The photosystems and electron transport enzymes of coupling membranes also transport protons in order to produce electrochemical gradients. The end result takes the form of membrane potentials ($\Delta\Psi$) or concentration gradients (ΔpH), which in turn provide energy sources and regulatory devices for cell and organelle functions.

If proton gradients across membranes are to be maintained by active transport processes, the membrane must present a sufficient barrier to proton diffusion. However, conductive pathways within the membrane are also required to direct protons to specific sites involved in membrane function. The focus of this review concerns these two aspects of passive proton transport: what is the nature of the barrier to proton diffusion across membranes, and how might protons be conducted within or across that barrier? As will be seen, these questions lead to more general considerations of ion and water permeation.

Key Words proton flux · lipid bilayers · membrane permeability · gramicidin · ion channels

Membrane Permeability to Ions Varies Widely

Three different membrane systems will be compared with respect to passive and active proton flux: liposomes, planar lipid membranes, and certain biological membranes. Liposomes are relatively simple, consisting of lipid bilayers in the form of vesicles. Because of the large surface areas available (~2200 cm^2 per mg of lipid dispersed as bilayer vesicles) flux of solutes even as relatively impermeant as sodium, potassium and chloride can be measured by radioactive tracers, various dyes, ion-sensing electrodes, and spin labels. Planar lipid membranes have much smaller areas available to flux (~1 mm^2) but have the advantage that ionic conductance can be measured across the membrane under very broad conditions of pH and ionic strength. Because of the small area, the conductance of typical physiological ions is low (10^{-9} S cm^{-2}) but can increase by several orders of magnitude if the membrane contains ionophores or channel structures. In "bare" planar lipid membranes, conductances have been measured for proton-hydroxide (Gutknecht, 1984, 1987) and relatively permeant ionic species such as tetraphenylphosphonium, tetraphenylboron, perchlorate and thiocyanate (Ketterer, Nuemcke & Läuger, 1971; Dilger et al., 1979).

The ionic conductance of biological membranes is well established and represents a useful comparison with proton-hydroxide flux. The conductive properties arise from ion-specific channels and non-

specific leaks. An important point is that the leaks produce membrane permeabilities to ions that are always orders of magnitude greater than those of lipid bilayers. For instance, the permeability of sodium in a liposome system is in the range of 10^{-12} cm/sec (Nozaki & Tanford, 1981), but in an erythrocyte membrane this value increases to near 5×10^{-9} cm/sec (Sze & Solomon, 1979) even though the red cell has no channels specialized for passive sodium transport. It follows that the bilayer is the primary barrier to free diffusion and that introduction of integral proteins into the bilayer provides not only channels, but also nonspecific conductance pathways which must be overcome by active ion transport.

Relatively little attention has been paid to the proton-hydroxide permeability of biological membranes. When permeability has been measured, it is typically orders of magnitude greater than expected from sodium-potassium permeabilities. For example, estimates of proton-hydroxide permeability of brush border membranes range from 0.006 to 0.3 cm/sec (Ives & Verkman, 1985; Verkman & Ives, 1986). Even in coupling membranes calculated permeabilities are near 10^{-3} cm/sec (Nichols & Deamer, 1980; Krishnamoorthy & Hinkle, 1984). The integration of such results into a more general understanding of proton permeability barriers, active proton transport, and membrane function is a central aim of this review.

Born Energy Considerations Do Not Account for Ionic Flux Across Lipid Bilayers

Why is the bilayer such a significant barrier to free diffusion of ions? When this question was first asked, the simplest approach was to assume that the bilayer interior was a uniform phase resembling hydrocarbon in dielectric properties. The assumption of uniformity permitted initial mathematical analysis according to Born energy considerations. Parsegian (1969) first attempted such analyses and showed that the Born energy required for a typical monovalent cation to leave water (dielectric = 78) and enter the hydrocarbon interior of a lipid bilayer (dielectric = 2) was in the range of 160 kJ mol^{-1}. These calculations assumed an ionic radius of 0.2 nm, representing the unhydrated crystal radius. If we assume a hydrated radius of 0.4 nm, the energy is still in the range of 80 kJ mol^{-1}. This represents a very substantial energy barrier, but Parsegian showed that the barrier can be greatly reduced by the presence of high dielectric defects or channels. An example to be discussed later is gramicidin, which contains a channel sufficient to accommodate a single chain of water molecules. This reduces the energy barrier so effectively that essentially free diffusion of ions occurs through the channel.

Are Born energy considerations sufficient to explain the bilayer barrier in an experimental system? This question was first raised by Hauser, Oldani and Phillips (1973) who measured the efflux of sodium from small unilamellar vesicles and compared the measured rate with rates calculated by estimating ionic solubility in the bilayer phase from Born energy. The measured rates were found to be too high by three orders of magnitude, leading to the suggestion that substantial conductive defects were appearing in the membrane. Hauser et al. proposed that the defects were produced by disruptive events when vesicles collided and fused, a process known to occur in small vesicles produced by sonication. However, in later studies with large unilamellar vesicles, measured sodium permeability was even higher, near 10^{-12} cm/sec (Nozaki & Tanford, 1981). Large vesicles resist fusion, which can only be induced under specialized circumstances (*see* Benz & Ellens, 1988, for review). Therefore the surprisingly high permeability of sodium in the large vesicles must result from fairly substantial transmembrane defects in the bilayer itself. Such defects presumably represent a rare extreme of a spectrum, which ranges from relatively common fluctuations just sufficient to let one or a few water molecules enter the bilayer, to strands or planes of hydrogen-bonded water extending into the bilayer, to the transmembrane ion-conducting defects described above. To give some idea of this range, one can readily calculate from measured fluxes that approximately 4,000 water molecules per second move through a membrane area equivalent to a single phospholipid molecule, while only one sodium ion crosses the same area in 70 hr (Deamer & Bramhall, 1986).

A second discrepancy is that anions are generally more permeant than equivalent cations. This is seen most clearly in comparisons of tetraphenylboron and tetraphenylphosphonium permeability, which are essentially identical except for the sign of their electrical charges, yet differ by factors of 10^3 to 10^4 in translocation kinetics across membranes (Flewelling & Hubbell, 1986*a*). In another example, chloride has a permeability coefficient near 10^{-11} cm/sec, in contrast to sodium or potassium permeabilities near 10^{-12} cm/sec. This again would not be expected from Born energy considerations, which treat positive and negative ions as equivalent.

Flewelling and Hubbell (1986*b*) have considered total contributions to the energy barrier faced by ions crossing membranes, including Born energy (W_B), image energy (W_I), dipole energy (W_D) and

neutral energy (W_N) and suggest that the energies are additive:

$$W_{TOTAL}(z) = W_B(z) + W_I(z) + W_D(z) + W_N(z)$$

where z is a function of position in the aqueous phase/bilayer system. The dipole energy is surprisingly important, and represents the chief discriminating factor in the higher permeability of anions relative to cations. Its primary effect is to increase the partitioning of anions into the bilayer by approximately 5000-fold, thereby reducing the activation energy of translocation from near 20 kcal/mol (TPP^+) to near 14 kcal/mol (TPB^-).

Finally, the conductive properties of untreated planar lipid membranes also indicate a nonuniform barrier to ionic current under certain conditions. For instance, conductance can occur in discrete jumps during lipid phase transitions (Antonov et al., 1980) and following pH shifts and voltage jumps (Kaufmann & Silman, 1983) as though substantial defects were permitting multiple conductance of ions. It is interesting that a computer model of lipid dynamics in bilayers also predicts that such transient defects will be present (Owenson & Pratt, 1984).

From this, we conclude that the hydrophobic interior of lipid bilayer membranes is not a uniform bulk phase with a uniform dielectric constant. Instead, the weight of evidence supports the existence of rare, transient fluctuations in lipid bilayer structure which permit ionic conductance at rates much greater than predicted by simple application of Born energy considerations.

Proton Flux is Anomalously High

Proton flux is a very general term and should first be defined as it will be used in this review. The basic measurements are made by monitoring decay of pH gradients across liposome membranes, or by measuring conductance in planar lipid membranes under conditions where no ions other than protons or hydroxide are available to carry current. There is no simple way to distinguish between the contributions of protons and hydroxide ions to the apparent flux, particularly at neutral pH ranges. Until this is clarified we will use the term *proton flux* to describe decay of pH gradients or movement of proton-hydroxide in response to membrane potentials, with the understanding that mechanisms may involve actual diffusion of protons and hydroxide ions, as well as proton translocation along hydrogen bonded chains. In the discussion to follow we will also consider the possibility that protons are transported by weak acid protonophores present as contaminants.

Nichols and Deamer (1978) first attempted to estimate proton permeability from measurements of the decay rate of pH gradients, and reported that the apparent permeability was much higher than expected from that of other monovalent ions. This measurement was confirmed and refined in later reports (Nichols et al.,1980; Nichols & Deamer, 1980; Deamer & Nichols, 1983), and it is now generally accepted that proton flux is anomalous, both in liposome systems and planar lipid membranes (PLM) (*see* Deamer (1987) and Gutknecht (1987*a*) for review). There are significant differences in liposomes and PLM which should be noted, however. First, proton flux is measured electrically in PLM systems, either as conductance under applied voltage, or development of membrane potentials from transmembrane proton concentration gradients. In liposomes, proton flux is measured indirectly, using various probe molecules to monitor decay of pH gradients. Examples include absorbance and fluorescence changes in dyes (Nichols et al., 1980; Clement & Gould, 1981*a*; Pohl, 1982; Elamrani & Blume, 1983; Barchfeld & Deamer, 1985; Pittarich & Lawaczeck, 1985) and spin label response to proton diffusion potentials (Cafiso & Hubbell, 1983; Perkins & Cafiso, 1986). Ion-sensitive electrodes can also be employed to measure pH shifts in the medium as proton flux takes place across liposome membranes (Nichols & Deamer, 1980; Rossignol, Grignon & Grignon, 1982) or planar lipid membranes (Gutknecht & Walter, 1981).

Another important difference is that most of the measurements made with planar lipid membranes involve bilayers containing decane or similar solvents as stabilizing agents, which are absent in liposome systems. The presence or absence of a solvent can have a major effect on bilayer properties. For instance, the conductance of a planar lipid membrane increases over 10-fold when bilayers with and without solvent are compared (Dilger et al., 1979).

Mechanisms of Proton Flux in Bilayers

Two alternative mechanisms for the anomalous proton flux have been suggested. The first is that the flux is mediated by traces of a weak acid protonophore in all lipid bilayers, perhaps arising as a product of oxidation or hydrolysis (Gutkneckt & Walter, 1981; Gutknecht, 1987*b*). The second is that the anomaly arises from some more fundamental process which is intrinsic to the physical properties of lipid bilayers, and involves transient formation of hydrogen bonded chains of water within the bilayer.

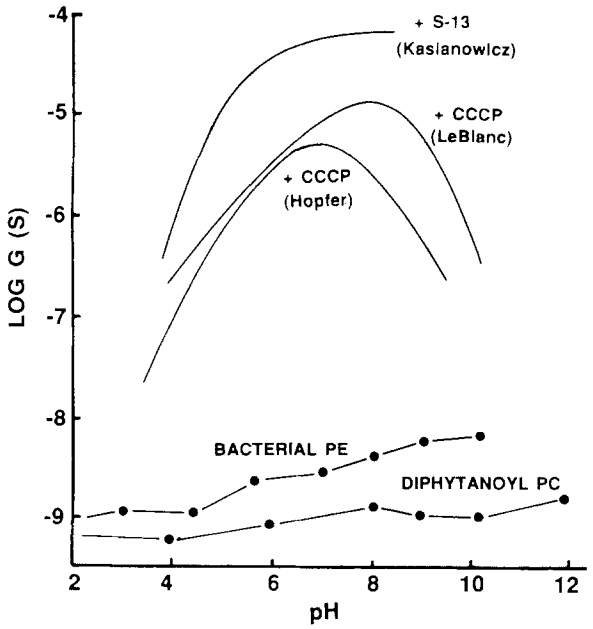

Fig. 1. Proton conductance in planar lipid membranes. The phosphatidylethanolamine (PE) and phosphatidylcholine (PC) data were redrawn from Gutknecht (1987). The upper line is drawn through data points given by Kasianowicz et al. (1987) showing the effect of protonophore addition on proton conductance. Earlier results for CCCP additions (Hopfer, Lehninger & Thomson, 1968; LeBlanc, 1971) are shown for comparison. The S-13 (0.1 μM) caused proton conductance to increase by 3 to 5 orders of magnitude, with a characteristic plateau above the pK of the protonophore

Gutknecht has tested the idea that some portion of the proton current in PLM is carried by weak acid contaminants acting as protonophores (*see* Gutkneckt, 1987*a* for review.). In support, proton conductance is highest in polyunsaturated phospholipids that are most likely to have oxidation damage, and lowest in relatively stable lipids such as diphytanoyl phosphatidylcholine. Furthermore, additions of simulated hydrolysis products such as phytanic acid (0.2 mole fraction) increases PLM conductance about 10-fold. As expected, bovine serum albumin reduces conductance. Phloretin is known to depress the dipole potential of lipid bilayer, and its addition reduces proton conductance, which would follow if anionic weak acids were involved in charge conduction. Gutknecht (1987*a*) has also shown that the water permeability of planar lipid bilayers does not vary significantly when different lipid compositions are compared, even though proton conductance may vary within those same membranes by two orders of magnitude. This result argues against a relationship between proton conduction mechanisms and water flux mechanisms.

In contrast, results from liposome systems do not support the protonophore hypothesis. For instance, serum albumin and fatty acid additions do not have marked effects on the decay of pH gradients in liposome systems (Deamer, 1987; Perkins & Cafiso, 1986) nor does phloretin inhibit flux (Perkins & Cafiso, 1986). When liposomes are put through a temperature-dependent phase transition, both water permeability and proton permeability increase by two orders of magnitude, arguing in favor of a role of water in the proton permeability changes related to gel/fluid phase transitions (Elamrani & Blume, 1983; Pitterich & Lawaczeck, 1985).

Despite these differences, the liposome and PLM model membrane systems agree on two important points: when proton permeability is calculated from proton conductance in PLM and liposomes at neutral pH ranges, the result is vastly higher than the permeability of other ions. Second, proton conductance is relatively independent of the concentration of the conducting species, whether protons or hydroxide ions (Gutknecht, 1984). This relative independence was noted in earlier work with liposomes over much narrower pH ranges of 2–3 units (Nichols & Deamer 1980; Cafiso & Hubbell, 1983). It is important to emphasize here, following Nagle (1987) that the observed independence of proton conductance and pH is a fundamental property of lipid bilayers. In this review we focus on proton flux (down concentration gradients) or conductance (down membrane potentials) and use permeability values only to compare different membrane systems at the same pH.

To illustrate the relative independence of proton conductance and pH, Fig. 1 shows results from planar lipid membranes (redrawn from Gutknecht, 1987*a*). Proton conductance of phosphatidylethanolamine and diphytanoylphosphatidylcholine membranes increased by less than 10-fold as the pH changed over 10 orders of magnitude. Figure 1 also illustrates the effect of the protonophore S-13 on conductance (results of Kasianowicz, Benz & McLaughlin, 1987) in which a characteristic plateau conductance occurs near the pK of the protonophore. The pure lipid membranes show only a modest increment in conductance with pH, arguing against a mechanism involving contaminating protonophores. However, the hypothetical protonophore could be relatively inefficient in conducting current. For instance, Gutknecht (1987*b*) has shown that when fatty acids are added to PLM, current is carried by the fatty acid anion. The flip-flop rate of the anion is rate-limiting, thereby reducing efficiency relative to other A^- protonophores by a factor of 10^5.

Table 1. Physical properties of water and ice relevant to proton flux mechanisms (modified from Eisenberg and Kauzmann, 1969)

	25°	−10°
Dissociation constant		
$K = C_H \cdot C_{OH}$	1.008×10^{-14}	—
$K = C_H \cdot C_{OH}/C_{H_2O}$	1.821×10^{-16}	2.40×10^{-23}
Ionic concentration (mol/liter)	1.004×10^{-7}	1.4×10^{-10}
Specific conductance (Ω^{-1} cm^{-1})	5.7×10^{-8}	—
Mobility of H^+ (cm^2/V · sec)	3.62×10^{-3}	75×10^{-3}
(For comparison, Na^+ and K^+ mobilities are 0.52×10^3 and 0.76×10^{-3})		
Mobility of OH^- (cm^2/V · sec)	1.98×10^{-3}	50×10^{-3}
For comparison, the mobility of Cl^- is 0.79×10^{-3})		
Rate constant dissociation (sec^{-1})	2.5×10^{-5}	3.2×10^{-9}
E_a of dissociation (kcal/mole)	15.5–16.5	22.5
Rate constant association liter/mole · sec)	1.4×10^{11}	8.6×10^{12}
Proton transfer rate liter/mole · sec)	1.1×10^{10} ($H_2O + H_3O^+$)	—
	6×10^9 ($H_2O + OH^-$)	—
Dielectric constant	78.54	95
Dipole moment	1.87 D	2.94 D
Diffusion coefficient (cm^2/sec)	2.4×10^{-3}	8×10^{-11}

What Underlies the Proton Conductance Anomaly?

If proton flux across liposome membranes cannot be explained as transport by contaminating protonophores, what mechanisms might account for such flux? Some pertinent physical properties of protons and water are summarized in Table 1. From these, proton conductance might be expected to differ from that of other ions in several ways.

1. Proton mobility in water is sevenfold higher than that of other biologically relevant cations (sodium, potassium) due to its ability to move along transient hydrogen bonded clusters of water molecules. This effect is greatly magnified in ice, in which protonic conductance predominates (Eigen, 1963).

2. Proton-hydroxide pairs are continuously produced by dissociation of water. This has the potential to provide conductive species at potential transport sites by a nondiffusional process.

3. The diffusional mobility of protons is affected by the presence of fixed or mobile buffer ions (Junge & McLaughlin, 1987).

4. It has been suggested that protons might have special conductive pathways along the surfaces of membranes (Kell & Morris, 1980; Haines, 1983; Teussie et al., 1985).

Each of these processes will now be considered in terms of their potential contributions to the overall magnitude of the proton flux anomaly.

The possibility of special conductive pathways along membrane surfaces has been tested experimentally by Gutman and Nachliel (1985) who used laser flashes to produce proton pulses from pyranine in lipid-water systems. The diffusion of protons back to the dye molecules could then be monitored by spectral changes and the effect of increasing lipid concentrations determined. If there were conductive pathways along membranes, it would be expected that proton diffusion would be markedly affected at higher lipid concentrations where membrane surface interactions would begin to dominate the water present.

No evidence for unusually high proton mobility near membrane surfaces was found. If anything, proton diffusion was inhibited several-fold near membrane surfaces, suggesting that surface water

structure was less conductive to protons than bulk phase water. We conclude that proton conductance along membrane surfaces does not contribute to the measured transmembrane flux.

With respect to fixed and mobile buffers, the contribution of such effects have been measured both in liposomes (Grzesiek & Dencher, 1986) and in bulk phase proton equilibration processes (Gutman & Nachliel, 1985; Junge & McLaughlin, 1987). The effective diffusion coefficients of protons are always lowered by the presence of buffers, an effect which, if anything, would tend to reduce the magnitude of the proton anomaly.

We can now go on to estimate potential contributions of the other factors. We are attempting to account for the difference between a typical proton-hydroxide permeability (10^{-6} cm/sec) measured at neutral pH ranges in large unilamellar vesicles (Perkins & Cafiso, 1986) and the permeability of potassium or sodium (10^{-12} cm/sec) observed under similar conditions (Nozaki & Tanford, 1981). We will first consider the ionic mobility of protons. How might a sevenfold greater ionic mobility account even in part for the enormous flux anomaly? One approach is to assume that no other effects contribute to proton flux other than the relative ability of a proton and potassium ion to strike a target represented by the transient defects assumed to be present in the bilayer. To a first approximation, the relative probability is proportional to the ratios of the ionic diffusional volumes of protons and potassium ions which intersect with a small target area representing a transient defect in the surface of a lipid bilayer. The ratio of diffusional volumes is approximately 7^3, or 350 to 1 for protons and potassium ions. (The equivalent ratio for hydroxide, which has an ionic mobility about twice that of chloride, is negligible.) Proton flux should therefore be greater than that of potassium flux by nearly 350 times, based solely on relative mobility of protons.

A second factor is that proton-hydroxide pairs are continuously being produced by hydrolysis of water, while other ions can only enter a target defect by physically diffusing to it. For instance, Kasianowicz et al. (1987) have shown that proton conductance by a protonophore is two orders of magnitude greater than expected from diffusional mobility of ions.

There is no reason to think that the two processes described above are other than additive, each contributing in part to the anomaly. When they are added, the ratio is near 500, leaving approximately four orders of magnitude still to be accounted for.

Hydrogen Bonded Chains of Water May Contribute to the Anomaly

When the magnitude of the proton flux anomaly was first noticed (Nichols & Deamer, 1980) it was suggested that a small fraction of the water in the bilayer might be associated through hydrogen bonding, thereby providing a conductance pathway unique to protons. Nagle and Morowitz (1978) first suggested that hydrogen bonded chains of amino acid side groups might form a wire-like conductive pathway (a "proton wire") within bilayers of biological membranes and perhaps contribute to certain membrane functions. Nagle and Tristram-Nagle (1983) extended this argument, and Nagle (1987) suggested the term "transient hydrogen bonded chains" (tHBC) to distinguish between the protein HBC and the water HBC proposed for bilayer proton conductance. We will use these terms here.

At first glance, a tHBC of water would seem to be highly implausible. How could relatively weak hydrogen bonding stabilize extensive associations of approximately 20 water molecules in the bilayer? Several developments now make this idea somewhat more palatable. As described earlier, the bilayer cannot be considered to be a nearly impermeable barrier to ions. Instead, substantial numbers of transient defects are required to account for the measured permeation of ions like sodium, potassium and chloride through the bilayer. Given a defect, it is not too difficult to consider that the water in the defect might be extensively hydrogen bonded for at least some fraction of time. This concept can also account for the constant proton conductance, and leads to testable predictions relative to kinetics of flux and the type of defect.

How much associated water might be necessary to produce sufficient hydrated defects to account for the proton flux anomaly? There have been several attempts to estimate this number, and the results are widely variant. Biegel and Gould (1981) measured proton flux in small unilamellar vesicles which contain approximately 4000 lipid molecules and confirmed that proton permeability was vastly greater than that of other cations. Using the solubility of water in alkanes as a guide, it was estimated that approximately 20–30 water molecules are present in the bilayer volume of a single liposome at any given time. Because 20 water molecules are required to span the thickness of a bilayer, it follows that much of the water present must spend some amount of time in a transmembrane-associated state, if tHBC cause the proton flux anomaly. This estimate, of course, is for the time-averaged water in the bilayer at equilibrium, and does not

take into account the possibility of rare transient defects due to fluctuations in the bilayer structure itself.

A second estimate used the gramicidin channel (Deamer, 1987). Assuming that the single strand of water molecules in the channel had properties similar to those of a tHBC, it was possible to ask how many channels would be required to account for the measured proton flux anomaly. Gramicidin was titrated into liposome preparations, and proton flux approximately doubled when its concentration reached the equivalent of one channel for 10^5 phospholipids, or six channels per liposome for vesicles 0.2 μm in diameter. If all the gramicidin added formed channels, the amount of water in the channels would represent about 7% of the water in the bilayer. However, the gramicidin dimers are in equilibrium with monomers, with a dimerization constant of 6×10^{13} mol^{-1} cm^2 (Veatch et al., 1975). From this it can be calculated that only 60% of the monomers form dimers under our conditions. This reduces the original estimate of water channels to 2–3% of total membrane water, still a surprisingly large value that probably represents an upper limit.

For comparison, Gutknecht (1987*e*) noted that at pH 2, the proton conductance of a gramicidin channel is in the range of 22 to 240 pS. From this value, it can be calculated that a single gramicidin-like channel in a planar lipid membrane of 0.02 cm^2 area would be capable of carrying sufficient current to account for the 1 nS/cm^2 "background" current of protons. However, this result is highly dependent on experimental conditions. For instance, Gutknecht (1987*a*) also showed that gramicidin proton conductance is proportional to [H$^+$], and it follows that 10^5 gramicidin channels would be required at pH 7 to carry the same current as at pH 2. The lipid/channel ratio would be equivalent to 2×10^9, and the amount of water would be in the range of one molecule in 10^6.

Another approach was suggested by J. Nagle (*personal communication*) who estimated the number of conducting tHBC required to account for the lower limit conductance of 10^{-9} S measured by Gutknecht in planar lipid membranes. The rate-limiting step was assumed to be Bjerrum turning defects moving along hydrogen bonded chains of water, resulting in a protonic mobility of 10^{-4} cm^2/V · sec, and these values were inserted into the flux equation shown below

$$G = \frac{n\mu e}{d} \text{ and } n = \frac{Gd}{\mu e}$$

$$= \frac{(10^{-9}\text{C/V} \cdot \text{sec} \cdot \text{cm}^2)(4 \times 10^{-7}\text{ cm})}{(10^{-4}\text{ cm}^2\text{/V} \cdot \text{sec})(1.6 \times 10^{-19}\text{ C})}$$

where G is conductance, n is the number of tHBC per cm^{-3}, μ is protonic mobility, e is the electronic charge, and d is the thickness of the bilayer nonpolar phase.

Solution of the equation for n, the number of tHBC, gave a result of 2.5×10^7 cm^{-3} bilayer volume, or 2×10^4 cm^{-2} bilayer area. The amount of water in tHBC was estimated by assuming 20 water molecules per defect, and a concentration of protonic charge in the defect equal to $e^{\beta U_{\text{BORN}}}$.

$$n_W^{\text{tHBC}} = n \cdot 20 \cdot e^{\beta U_{\text{BORN}}} = 3 \times 10^4 n = 10^{12}\text{ cm}^{-3}.$$

This was then compared with the total amount of water in the same volume (3.3×10^{18}/cm^3) which can be estimated either by extrapolating from bulk phase solubility of water in hydrocarbons or from the factor by which a bilayer reduces transmembrane diffusion of water (10^{-4})

$$\frac{n_W^{\text{tHBC}}}{n_W^{\text{TOTAL}}} = \frac{10^{12}\text{ cm}^{-3}}{3.3 \times 10^{18}\text{ cm}} \cong 10^{-6}.$$

The result suggests that the proton conductance anomaly could be accounted for by only 1 water in 10^6 molecules of water in the bilayer phase, a result similar to that estimated by extrapolating the gramicidin proton conductance measured by Gutknecht (1987*a*).

Miller (1987) used a different approach, in which water concentration in the bilayer was first estimated from the relationship

$$P_w = 18\, C_w \cdot D_w/d$$

where P_w is the permeability coefficient of water (2×10^{-3} cm/sec), D_w is the diffusion coefficient of water in bilayers estimated from microviscosities ($1.5 - 3 \times 10^{-7}$ cm^2/sec) and d is the thickness of the bilayer nonpolar phase (2.6×10^{-7} cm). This relationship can be solved for the water concentration (C_w) in the bilayer which turns out to be 1.2 ± 0.4 moles/cm^3, or about 0.1 M. Miller then calculates association constants of water for various sizes of hydrogen bonded aggregates in a nonpolar medium and estimates that the number of water chains ranges between 4×10^{11} to 10^{12}/cm^2.

This value is much greater than Nagle's estimate of 10^6/cm^2, in part because of the calculated water concentration in the bilayer phase (0.1 M). This high concentration arises from the value chosen for the water diffusion coefficient, which is lower by two orders of magnitude than other estimates (*see* Fettiplace & Haydon, 1980). Furthermore, some experimental observations argue

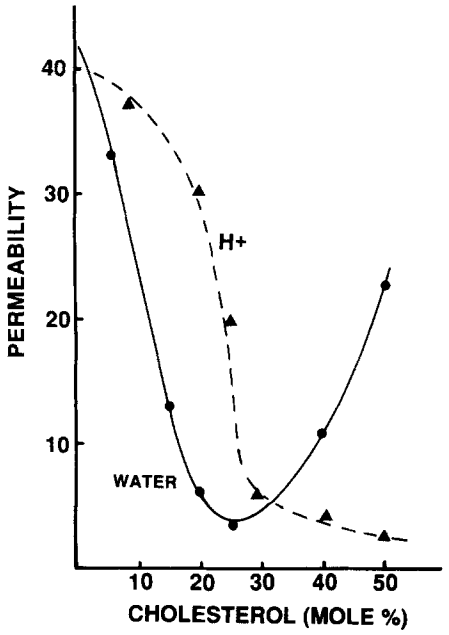

Fig. 2. Effect of cholesterol additions on water and proton permeability of liposomes. Water data is taken from Carruthers and Melchior (1983) and proton data from Li et al. (1988). The lipids were dimyristoyl phosphatidylcholine (water) and egg phosphatidylcholine (protons). The permeability scale is for the proton data, and is given in units of (cm sec^{-1}) $\times$ 10^{-4}. The water permeability was measured by light scattering changes, and the results are normalized to the proton permeability scale in arbitrary units. Note that cholesterol additions up to 30 mole % decreased both water and proton permeability

against such a high water content of the bilayer. For instance, the concentration of water in water-saturated hydrocarbons is near 0.005 M, a value which Cass and Finkelstein (1967) have shown to be consistent with the measured permeability coefficient if water diffuses across the bilayer as monomers.

The fraction of water in the form of tHBC is relevant to an observation of Gutknecht (1987*a*) who showed in planar lipid membranes that proton conductance varied as much as 100-fold among different types of lipids, while water permeability, measured as flux of tritiated water, was unchanged. If only a small fraction of water is present as tHBC, with most of the water permeating as monomers, there is no necessary correlation between water permeability and proton permeability. Instead, specialized defects able to transport protons could exist quite apart from water transport processes.

However, there is also evidence that water and proton permeation are correlated under certain conditions. For instance, Elamrani and Blume (1983) compared proton and water permeability of liposomes above and below a phase transition temperature and found that both parameters increased by two orders of magnitude in the fluid phase. The effect of cholesterol on water and proton permeation is also pertinent. Figure 2 compares the water permeability of dimyristoyl phosphatidylcholine vesicles (Carruthers & Melchior, 1983) and proton permeability of egg phosphatidylcholine vesicles (Li et al., 1988) as cholesterol was titrated from 0 to 50 mole%. Both water and proton permeability are reduced to similar extents, even though the results were obtained with phospholipids having quite different acyl chain compositions.

Kinetics of Proton Flux in tHBC Can Be Modeled Mathematically

Nagle (1987) has derived equations describing proton flux under different assumptions regarding the flux mechanism. The constant conductance measured at vastly different concentrations of potential conductive species (H^+ and OH^-) cannot be fitted by any of the usual flux equations derived from Fick's laws of diffusion. Therefore the most plausible hypothesis for the anomalous proton flux across lipid bilayers involves some form of transmembrane defect containing transient hydrogen bonded chains of water.

Nagle proposes three types of defects which can be described mathematically. In the first (model A) the defect lasts long enough to conduct several protons during its lifetime. In model B the defect is relatively short lived, so that at most a single proton is conducted. The third defect involves two partial chains of associated water penetrating the bilayer, one carrying a proton, the other a hydroxide (model C). The analysis assumes that a proton equivalent is conducted by recombination of the two ions to form water when the two chains touch, as suggested earlier (Deamer & Nichols, 1985). The observed independence of G and pH follows clearly from model C, since the proton conductance step occurs only when chains carrying both a proton and a hydroxide happen to touch. Models A and B can account for constant conductance with pH if a step other than proton translocation is rate limiting. In model A, the diffusion of the turning defect is taken to be rate limiting, and in model B the formation of conductive channels is rate limiting. The equations governing the three models are given in Table 2.

The three models describe experimentally testable systems, in which plots of flux against membrane potentials or pH gradients give curve shapes ranging from sublinear to superlinear (Fig. 3). How do experimental results correspond to the alternative equations? The relationship of ionic current to imposed driving forces has long been of interest in

Table 2. Equations relating proton flux to driving force (Nagle, 1987)

Model	ΔpH	ΔΨ
A	$J = J_o \tanh(\beta\delta/2)$ (sublinear)	$J = J_o g(\delta) \sinh(\beta\delta/2)/\cosh(\beta\delta f/2)$ (superlinear)
B	$J = J_o \tanh(\beta\delta/2)/2$ (sublinear)	$J = J_o(e^{\beta\delta} - 1)/[(e^{\beta\delta f} + 1)(e^{\beta\delta(1-f)} + 1)]$ (sublinear)
C	$J = J_o \sinh(\beta\delta)$ (superlinear)	$J = J_o h(\delta) \sinh(\beta\delta/\cosh[\beta\delta 1 - f)/4])^2$ (superlinear)

membrane bioenergetics, and nonohmic behavior of membranes was observed by Hodgkin and Huxley as early as 1952. (*See* Haydon & Hladky (1972) for review.) Because of the general interest in ohmic and nonohmic ion conductance, and the relationship to proton flux mechanisms, other investigators have addressed this question. Some representative plots are shown in Fig. 4. For clarity, in most of the plots only the lines fitted to the data points by the authors are shown. Furthermore, in order to aid in comparing the results, all original flux figures have been converted to the same units, with details provided in the legend. Figure 4*A–E* shows proton flux across membranes driven by pH gradients, and Fig. 4*F–I* represent flux driven by membrane potentials. The latter were established in the vesicle systems by potassium gradients, with potassium ion made selectively permeable by valinomycin.

Figure 4*A* shows a result with PC:PA liposomes. We consistently find nonohmic, superlinear relationships when proton flux is plotted against the magnitude of the pH gradients. However, if a protonophore is present (Fig. 4*B*) the flux becomes linear with delta pH. (Valinomycin and potassium were present in both instances to prevent proton diffusion potentials from developing.) Three other investigations, using different lipid systems, do not agree with this result, instead reporting a linear relationship (Kell & Morris, 1980; Krishnamoorthy & Hinkle, 1984; O'Shea et al., 1984). Examples are shown in Fig. 4*C* and *D*. On the other hand, Verkmann (1987) observed superlinearity in a brush border membrane vesicle system (Fig. 4*E*).

Krishnamoorthy and Hinkle (1984) and O'Shea et al. (1984) also measured proton flux driven by membrane potentials across liposome membranes, and the results are shown in Fig. 4*F* and *G*. Under these conditions the plots are superlinear, but become linear with addition of protonophore. It is interesting to compare these observations with similar measurements in biological membranes. Figure 4*H* shows Krishnamoorthy and Hinkle's results for mitochondrial proton flux. The mitochondrial membranes appear to be approximately 30 times more permeable to protons ($P = 1.6 \times 10^{-3}$ cm/sec) than liposomes composed of mitochondrial lipids ($P = 5.2 \times 10^{-5}$) which in turn have twice the permeability of soy asolectin vesicles ($P = 1.9 \times 10^{-5}$). The plot of proton flux as a function of membrane potential is superlinear, similar to the results obtained with mitochondrial phospholipid. Verkman (1987) described similar experiments with brush border membrane vesicles (BBMV). His results for pH gradients are summarized in Fig. 4*E* and show a clear superlinearity, as described earlier. When the proton flux is driven by a membrane potential, the result was interpreted by the author to be linear (Fig. 4*I*). However, the points hint at superlinearity and could as easily be fitted to an exponential equation.

Finally, Fig. 4*J* shows Verkman's results for proton flux measured at different pH ranges. Significantly, although the biological membranes are three orders of magnitude more permeable to protons than lipid bilayers, they show the same constant proton conductance as pH is varied.

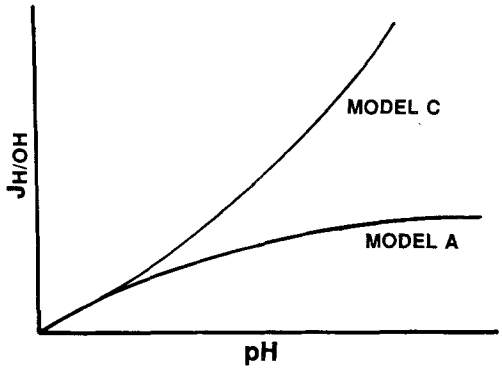

Fig. 3. The lines show computer plots of the sublinear and superlinear relationships between ΔpH and proton flux for models A and C of Nagle (1987). (*See* text for details.) Model A is strongly sublinear when proton flux is driven by a pH gradient, but can be superlinear for flux driven by a membrane potential. Model C is superlinear in both cases, and model B is sublinear (*not shown*)

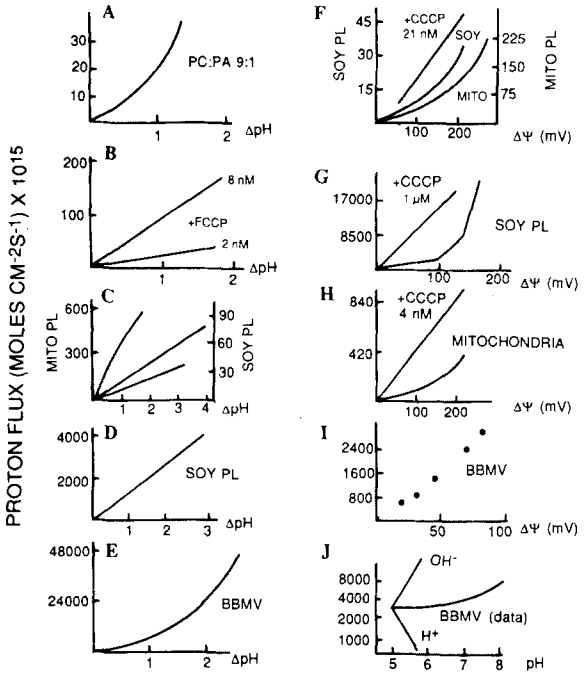

Fig. 4. Proton flux as a function of pH gradients and membrane potential has been measured by several investigators, and examples are shown here. For clarity, most data points have been omitted, and proton flux was expressed as moles cm^{-2} sec^{-1}. Lipid bilayer areas were calculated assuming an area of 2200 cm^2 per mg lipid, and membrane areas were approximated by finding the area occupied by a given weight of lipid + protein spread to a thickness of 8 nm. For mitochondrial membranes this value was 1600 cm^2 per mg protein (assuming 25% lipid dry wt) and for brush border membrane vesicles (BBMV) the value was 2500 cm^2 per mg protein, assuming 50% lipid dry wt. (*See* Branton and Deamer (1972) for a detailed description of this method.) (*A* and *B*). Large unilamellar liposomes were prepared from egg phosphatidylcholine (PC) and egg phosphatidic acid (PA) in 9 : 1 mole ratios, and proton flux was measured by a pyranine dye method (Deamer, 1987). Valinomycin was present to release diffusion potentials. Under these conditions, the plot of proton flux against pH gradient was superlinear, but became linear if a protonophore (FCCP) was added. Linear plots were obtained by Krishnamoorthy and Hinkle (1984) and O'Shea et al. (1984) with soy phospholipid and mitochondrial lipid (*C* and *D*). *E* shows data for brush border membrane vesicles (Verkman, 1987). Despite much higher permeability, proton flux was superlinear with pH gradient. Krishnamoorthy and Hinkle (1984) and O'Shea et al. (1984) also measured proton flux driven by membrane potentials, which were established by valinomycin and potassium gradients. Under these conditions plots of proton flux against membrane potential were superlinear (*F* and *G*) but became linear with protonophore addition. Similar results were obtained by Krishnamoorthy and Hinkle with proton flux across mitochondrial membranes (*H*). *I* shows an equivalent result from BBMV (Verkman, 1987), but experimental error in the data points was too large to permit a choice between linear and superlinear functions. *J* shows proton flux in BBMV at constant driving force, but over a range of pH values (Verkman, 1987). As in liposomes and PLM, the flux is relatively independent of pH, even though it is greater by three orders of magnitude. The lines labeled H^+ and OH^- show the expected results if only protons (H^+) or hydroxide anions (OH^-) were carrying the current

In summary, none of the curves is sublinear, and we can exclude models that lead to sublinear plots: model B for proton flux driven either by membrane potentials or pH gradients, and model A for proton flux driven by pH gradients. However, different membrane systems produced conflicting results with respect to superlinear plots, and further work will be necessary to resolve the discrepancies.

A related model describes nonohmic behavior for ions other than protons. This is based on earlier observations of Hladky (1974) who showed that an ionic current I crossing a membrane with a trapezoidal energy barrier can be described by

$$I = G_o bu \sinh(u/2)/\sinh(bu/2)$$

where b is the fraction of membrane spanned by the top of the trapezoid, and $u = F\Delta\Psi/RT$. This result produces superlinear plots under appropriate conditions. Krishnamoorthy and Hinkle (1984) found that their results shown in Fig. 4*H* can be fitted by an expression derived from the Hladky equation, of the form

$$\frac{J_{H/OH}}{J^{125}_{H/OH}} = \frac{u}{u^{125}} \cdot \frac{\sinh(u/2)/\sinh(bu/2)}{\sinh(u^{125}/2)/\sinh(bu^{125}/2)}$$

where $b = 0.6$ and J_{125} is the flux at 125 mV.

Gramicidin is a Model Proton Wire

Gramicidin A is a pentadecapeptide (mol wt 1882), which has the capacity to produce ion-conducting channels in lipid bilayers. A molecular structure proposed by Urry et al. (1971) has recently received strong supporting evidence (*see* Cornell (1987) for a review). It is now reasonably certain that the channel is produced when two gramicidin molecules form head-to-head hydrogen bonded pairs that span the membrane. The channel itself contains a single file of water, probably in the range of 9–12 molecules in length (Levitt, Elias & Hautman, 1978). Channel lifetime depends very much on the lipid composition and ranges from milliseconds to minutes. During the time that the channel is open, the conductance is 15 pS, equivalent to 2×10^6 ions per second.

In their original work, Myers and Haydon (1972) noted that proton conductance in the gramicidin channel was much greater than expected, and suggested that protons may be hopping along hydrogen bonded strands of water. This conjecture was supported by the work of Levitt et al. (1978) who showed that potassium and sodium flux through the channel produced a streaming poten-

tial, due to water being pushed through. However, proton flux did not produce a streaming potential, consistent with the idea that protons move along associated hydrogen bonds. Clement and Gould (1981*b*) and more recently Krishnamoorthy (1986) extended gramicidin studies to liposome systems and demonstrated that the channel is well able to conduct protons across liposome membranes as well.

We have compared proton and potassium flux through the channel in liposome membranes at neutral pH ranges, by titrating gramicidin into the liposomes and determining how much incremental flux was produced per channel. The number of ions per second passing through a single channel was then calculated. In one experiment, this number was 10 protons and 230 potassium ions per second. However, the proton concentration difference driving the flux was $10^{-6.5}$ M, while the potassium concentration difference was 0.4 M. Assuming that potassium flux is linearly proportional to its concentration, extrapolation gives a value of 1.8×10^{-4} potassium ions per second at $10^{-6.5}$ M potassium. The proton/potassium flux ratio at the same concentration is thus 5.5×10^4, showing that a proton flux anomaly also appears in a known tHBC, albeit not as large as that measured in lipid bilayers.

Although gramicidin A apparently conducts protons along hydrogen bonded chains of water, it differs from the hypothetical tHBC of lipid bilayers in one significant respect. Gutknecht (1987*b*) measured proton conductance across gramicidin-containing planar lipid membranes over a range of pH values, from 4.2 to 8.5. Conductance was found to be directly proportional to hydrogen ion concentration, in contrast to the relative independence of conductance and pH in pure lipid systems. Gramicidin A is known to be a highly specific cation-conducting channel. If hydroxide anion is unable to partake in translocation of proton equivalents along the tHBC, it follows that conductance would depend entirely on proton concentration.

Gramicidin might at first seem to be a highly evolved channel produced by bacterial species for its antibiotic effect. However, recent results indicate that it may be relatively simple to obtain similar specificity with other peptides. Lear, Wasserman and DeGrado (1988) have synthesized several peptides from leucine and serine, then tested their abilities to produce ion conducting channels in planar lipid membranes. It was found that a 21-residue peptide H_2N-(LSSLLSL)$_3$-$CONH_2$ formed channels with ion permeability properties resembling those of the acetylcholine receptor. A 14-residue peptide containing two instead of three repeating sequences did not produce stable channels. The channels in 0.5 M KCl had lifetimes in the range of 3–8 msec and cation conductances of 70 pS (4.3×10^7 ions/sec) at 100 mV potentials. Their dimensions were measured by substituting large organic ions, and were found to be approximately 0.8 nm in diameter. Significantly, the peptide H_2N-(LSLLLSL)$_3$-$CONH_2$ with one serine replaced by a leucine formed channels that were highly specific for protons. Their conductance was 120 pS, with lifetimes in the range of 1 msec. No cations other than the protons produced measurable conductances, even at concentrations between 2.5 M KCl to 10 M LiCl. Other considerations, including computer modeling, suggest that the proton channel is a trimeric or tetrameric aggregate of alpha helices, while the cation-conducting channel is hexameric or larger.

Are Water Wires Present in Biological Membranes?

A significant question is whether anything resembling a water wire is present in biological systems. Several possible candidates include the pathway by which protons reach the pump site in bacteriorhodopsin, the anion channel of erythrocytes, and the F_o subunit of the ATP synthase in coupling membranes. Attempts to demonstrate a proton-selective channel in BR have generally been disappointing. There are no obvious wire-like configurations of peptides resembling those suggested by Nagle and Morowitz (1978) and molecular structures deduced from diffraction patterns do not leave much room for even a single line of water molecules. However, Konishi and Packer (1978) presented preliminary evidence consistent with a proton channel in the membrane form of bacteriorhodopsin. Supportive evidence has recently been obtained by Denscher, Burghaus & Grzesiek (1988) who observed that bacteriorhodopsin in which the retinal has been removed is significantly more permeable to protons than the native protein.

Probably the most likely candidate is the F_o subunit, which clearly has a proton transport function. Lill et al. (1986) and Schoenknecht et al. (1986) have measured proton flux rates through this channel, and after correcting for the large fraction (~97%) of channels that do not conduct proton current, the unit conductance was estimated to be 169 fS at an external pH of 7.5, and 100 mV driving potential. This is too fast to be accounted for by simple diffusion to the channel, but can be explained by the hydrolysis mechanism discussed earlier (Kasianowicz et al., 1987). The conductance is equivalent to approximately 10^5 protons per chan-

Table 3. Comparative conductance values for single ion channels

Channel	G (pS)	Ions/sec	Conditions	Reference
Gramicidin	220	13×10^7	H^+, PLM, pH 2	a
Gramicidin	2.5	—	H^+, Thylakoids	b
Gramicidin	0.06	36×10^3	H^+, PLM, pH 5	c
Gramicidin	0.21×10^{-3}	130	H^+, Liposomes, pH 7	d
Gramicidin	0.016×10^{-3}	10	H^+, Liposomes, pH 6.5	e
Peptide	120	—	H^+, PLM, 100 mV	f
CF_o	0.17	10^5	H^+, Thylakoids	g
CF_o	1.1	—	H^+, Thylakoids	h
Defect	2–5	—	Na^+, PLM, 110 mV	i

References: a) Hladky & Haydon, 1972; b) Lill et al.,1987; c) Eisenman et al., 1980; d) Krishnamoorthy & Hinkle, 1984; e) Deamer, 1987; f) Lear et al., 1988; g) Schoenknecht et al., 1986; h) Lill et al., 1987; i) Kaufmann & Silman, 1983.

nel per second, which is more than sufficient to accommodate even the highest ATP synthesis rates during photophosphorylation.

How does proton conductance of CF_o compare with that of other proton-transporting channels? This is not an easy comparison to make, in that measurements with model membrane systems are made with known concentration gradients or driving membrane potentials (Table 3). However, Lill et al. (1987) were able to estimate the single-channel proton conductance of gramicidin by adding it to thylakoid membranes, and obtained a value of 2.5 pS. Under the same conditions the single-channel conductance of CF_o was 1.1 pS, a remarkably good correspondence when the underlying assumptions and potential experimental errors are taken into account.

Concluding Remarks

The permeability barrier of lipid bilayers to ions is considerably more complex than described by a simple layer of hydrocarbon chains in which solutes are dissolved according to partition coefficients and Born energy considerations. Instead, there appears to exist a spectrum of defects in the bilayer, which are best described in terms of the amount of water they contain. Most common are defects that permit individual water molecules to enter the hydrophobic phase, while at the other extreme are rare, larger defects that permit flux of hydrated ions such as sodium.

The weight of evidence from both biological and model membranes indicates that passive proton translocation driven by concentration gradients or membrane potentials is anomalously high relative to other cations. The flux cannot be due solely to protonophoric transport by trace contaminants, although this may occur to varying extents in different membrane systems. To account for the anomalous flux, we have suggested that the mechanism involves some form of transport along transient hydrogen bonded chains of water present in the rare defects described above. The flux does not appear to follow traditional diffusion theory, but instead is essentially independent of the concentrations of protons and hydroxide ions. This observation suggests three different conductive mechanisms, which are presently being tested. The results so far exclude any mechanism that produces sublinear plots of proton flux against driving force (pH gradients or membrane potentials).

These considerations have implications for biological membrane function. For instance, hydrogen bonded chains of water are present in the gramicidin channel, and anomalously high proton flux through the channel has been demonstrated. Direct comparisons of single-channel conductance by gramicidin and CF_o in thylakoid membranes produce very similar results. We suggest that hydrogen bonded chains of water are plausible conductive pathways in membrane proteins involved in proton transport.

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