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MINI-REVIEW

Proton Permeation of Lipid Bilayers

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Abstract

Proton permeation of the lipid bilayer barrier has two unique features. First, permeability coefficients measured at neutral pH ranges are six to seven orders of magnitude greater than expected from knowledge of other monovalent cations. Second, proton conductance across planar lipid bilayers varies at most by a factor of 10 when pH is varied from near 1 to near 11. Two mechanisms have been proposed to account for this anomalous behavior: proton conductance related to contaminants of lipid bilayers, and proton translocation along transient hydrogen-bonded chains (tHBC) of associated water molecules in the membrane. The weight of evidence suggests that trace contaminants may contribute to proton conductance across planar lipid membranes at certain pH ranges, but cannot account for the anomalous proton flux in liposome systems.

Two new results will be reported here which were designed to test the tHBC model. These include measurements of relative proton/potassium permeability in the gramicidin channel, and plots of proton flux against the magnitude of pH gradients. (1) The relative permeabilities of protons and potassium through the gramicidin channel, which contains a single strand of hydrogen-bonded water molecules, were found to differ by at least four orders of magnitude when measured at neutral pH ranges. This result demonstrates that a hydrogen-bonded chain of water molecules can provide substantial discrimination between protons and other cations. It was also possible to calculate that if approximately 7% of bilayer water was present in a transient configuration similar to that of the gramicidin channel, it could account for the measured proton flux. (2) The plot of proton conductance against pH gradient across liposome membranes was superlinear, a result that is consistent with one of three alternative tHBC models for proton conductance described by Nagle elsewhere in this volume.

Key Words: Proton; permeability; lipid bilayer; water; gramicidin; deuterium oxide.

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Introduction

Two supramolecular structures are responsible for most membrane functions in the living cell. The first is the lipid bilayer, which acts both as a structural matrix and barrier, and the second includes a variety of lipid–protein complexes which mediate important membrane functions such as passive and active ion translocation, light transduction, and coupling of electron transport to ATP synthesis. Many such functions require ion gradients to be maintained against leaks, and therefore the permeability of the barrier is a significant aspect of membrane-related activities. This review will consider the barrier function of the lipid bilayer, and more specifically its permeability to protons and other cations.

In the early 1960s, liposomes and planar lipid bilayers were introduced as model membrane systems (Bangham *et al.*, 1965; Mueller *et al.*, 1962). The advantage of such systems is that solute flux can be readily monitored by a variety of techniques including ion-selective electrodes, radioactive tracers, and fluorescent dyes in liposomes, and electrical conductance across planar lipid bilayers. Permeability coefficients calculated from such measurements provided the first convincing evidence that the lipid bilayer moiety of biological membranes represents the primary barrier to free diffusion of solutes.

Given a barrier function, it is important to determine the extent of leakage through defects in the barrier, and the effect of induced leaks on membrane function. The significance of such measurements in biological membranes is discussed by Verkman (this volume) and is clearly illustrated by coupling membrane systems such as the prokaryotic plasma membrane, or mitochondrial and thylakoid membranes, in which modest increments in permeability can lead to serious disruption of membrane function. For example, the barrier is sufficient to maintain a protonmotive force of nearly 200 mV, essential for ATP synthesis, but if the permeability of such membranes is increased by additions of ionophores, their ion transport systems are unable to maintain gradients against the induced leak, and coupled reactions are inhibited.

From such results, it has generally been assumed that lipid bilayer permeability to all ions is sufficiently low so that it can be ignored. This expectation was confirmed in early experiments with planar lipid membranes, in which resistance to ionic conductance was found to be very high (Mueller and Rudin, 1967). Similarly, sodium ion concentration gradients were maintained with half-times of decay measured in weeks in small unilamellar liposomes (Hauser *et al.*, 1973) or hours in large unilamellar liposomes (Nozaki and Tanford, 1981). From the latter measurements, permeability coefficients of 10^{-12} cm s^{-1} were determined, which can be compared with values around 10^{-3} cm s^{-1} for water permeation of lipid bilayers. To give

some perspective on the physical significance of these values, it can be calculated from the above permeabilities that, in a 0.1 M sodium chloride solution, about half a million water molecules diffuse past a given phospholipid every second, and one sodium ion per month.

Because sodium and potassium permeabilities are so low, the first measurements of lipid bilayer permeability to protons were surprising and controversial when coefficients in the range of 10^{-5} to 10^{-4} cm s^{-1} were reported, orders of magnitude larger than those of other cations (Nichols and Deamer, 1980; Nichols *et al.*, 1980; Deamer and Nichols, 1983). The anomalous permeability of protons represents a central theme here, and several points should be immediately clarified. First, it is important to note that the anomaly arises from comparative measurements necessarily made with vastly different concentration gradients driving the flux. For instance, in a liposome model system, proton flux might be measured by the decay rate of a buffered pH gradient, say from pH 6.5 to 7.5, so that the driving concentration is near 0.1 μM. The proton flux is typically in the range of 10^{-15} mol cm^{-2} s^{-1}. When this is divided by the driving concentration (10^{-10} mol cm^{-3}), a permeability coefficient in the range of 10^{-5} cm s^{-1} is obtained. Measurements of potassium flux under similar conditions would typically be 10^{-16} mol cm^{-2} s^{-1}, driven by a concentration of 10^{-4} mol cm^{-3} (0.1 M), and the permeability coefficient is then 10^{-12} cm s^{-1}. Because the fluxes of protons and potassium vary only by a factor of 10, the difference in permeability coefficients arises largely from the difference in concentrations driving that flux. It is a reasonable assumption that if a potassium gradient of 0.1 μM was used, equivalent to that of protons at pH 7, the same difference would result. However, this experiment has not been carried out because of the technical difficulty in monitoring flux of 0.1 μM cations other than protons.

The above considerations are relevant to the function of coupling membranes: if protons are so permeant, how can such membranes maintain functional proton gradients? This was answered in part by an early paper of Mitchell and Moyle (1967), who showed that the proton *conductance* of mitochondrial membranes was in the range of 0.45×10^{-6} S cm^{-2}, no greater than that of other ions. However, as noted above, permeation of common monovalent cations (sodium, potassium) is driven by concentration gradients ranging around 0.1 M, while proton permeation is driven by gradients in the 0.1 μM range. Thus, if a permeability coefficient is calculated from the Mitchell and Moyle data (Nichols and Deamer, 1980), it is approximately 10^{-3} cm s^{-1}, significantly greater than values from lipid bilayers. It follows that membranes can have a high intrinsic permeability to protons, but the concentration is so low that conductance is negligible in terms of membrane function.

A second consideration is that flux of both protons and hydroxide ions can contribute to the decay of a pH gradient. In earlier work we introduced the term net proton-hydroxide permeability in order to include the potential contributions of both ionic species (Nichols and Deamer, 1980):

$$P_{net} = P_{H+} + P_{OH-}$$

It is unlikely that the hydroxide anion *per se* is contributing, since there is no reason to expect it to differ significantly from other anions such as chloride, which has a permeability coefficient near 10^{-11} cm s^{-1} (Papahadjopoulos *et al.*, 1972; Hauser *et al.* 1973). However, permeation of hydroxide equivalents can occur as translocation of proton defects, so that hydroxide concentrations must be taken into account in calculations of H^+/OH^- flux and permeability, particularly at alkaline pH ranges. For simplicity, the terms proton flux and proton permeability will be used here to refer to the process by which pH gradients decay or protons conduct current across bilayers under applied voltages, with the understanding that hydroxide equivalents may contribute to the net flux in some manner.

It should also be understood that the term proton permeability can be misleading, in that the conductance mechanism does not involve classic diffusion of particles through channels. Instead, the conductance, at least in lipid bilayers, is relatively constant with pH, so that calculated permeability coefficients are not intrinsic to the membrane, but instead depend on the conditions under which proton flux is measured. However, proton permeability coefficients do provide useful comparative information when measured under the same conditions of driving force (either a pH gradient or voltage) and make clear the vast difference between protons and other ions which is not immediately apparent from simple measurements of conductance.

With these caveats, it is useful to list some examples of proton permeability coefficients which have been obtained by a number of laboratories, and compare them to values for other ions (Table I). There is agreement that lipid bilayers have an anomalously high permeability to protons compared with other monovalent cations. However, the measurements of proton permeability coefficients show a large variance between laboratories, with perhaps four orders of magnitude separating the highest and lowest values. Perkins and Cafiso (1986) made an extensive study of different liposome preparations, and determined that most if not all of the variation could be understood as actual differences among physical and chemical properties of the lipids and conditions of measurement. It appears that there is convergence on values ranging between 10^{-6} to 10^{-5} cm s^{-1} for planar lipid membranes and large unilamellar liposomes composed of typical lipid mixtures and measured at neutral pH ranges. This will be taken as a reasonable value for the purposes of the review. If we compare it to sodium/potassium permeability

Table I. Examples of Ionic Permeability Coefficients for Lipid Bilayers Measured Near pH 7[a]

Bilayer model	Permeability coefficient (cm/s)	Reference
Protons		
Large liposomes		
Egg PC:PA 9:1	1.4×10^{-4}	Nichols and Deamer (1980)
Plant phospholipid	1.4×10^{-4}	Rossignol *et al.* (1982)
DMPA	10^{-5} (gel) to 10^{-3} (fluid)	Elamrani and Blume (1983)
POPC	7×10^{-7}	Perkins and Cafiso (1986
DAPC	1.8×10^{-5}	Perkins and Cafiso (1986)
Small liposomes		
PC	1.4×10^{-4}	Pohl (1982)
POPC	5.9×10^{-7}	Perkins and Cafiso (1986)
Planar lipid bilayers		
Bacterial PE	1×10^{-5}	Gutknecht (1987)
Diphytanoyl PC	4×10^{-6}	Gutknecht (1987)
PE + BSA wash	7×10^{-7}	Gutknecht (1987)
Sodium		
Small liposomes	10^{-14}	Hauser *et al.* (1973)
Large liposomes	10^{-12}	Nozaki and Tanford (1981)

[a]Abbreviations: PC, phosphatidylcholine; PA, phosphatidic acid, POPC, 1-palmitoyl-2-oleoylphosphatidycholine; DMPA, dimyristoyl phosphatidic acid; DAPC, diarachidonoyl-phosphatidylcholine; PE, phosphatidylethanolamine; BSA, bovine serum albumin; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazine; pyranine, 8-hydroxy-1,3,6-pyrenetrisulfonate.

coefficients of 10^{-12} cm s^{-1} for large vesicles prepared under similar conditions, the proton permeability anomaly is about six to seven orders of magnitude. This represents a primary question to be examined here: What mechanism gives rise to the unexpected difference?

A second question concerns the remarkable fact that proton conductance is surprisingly constant as pH is varied. This has been remarked upon previously (Nichols and Deamer, 1980; Cafiso and Hubbell, 1983; Perkins and Cafiso, 1986) and was investigated in some detail by Gutknecht (1984). The constancy is unique to proton flux, and surely represents an important clue to the underlying mechanism.

The third question to be examined is related to Born energy (Born, 1920) considerations of ion flux across membranes, and to water permeation mechanisms. Parsegian (1969) calculated Born energies required for a typical ion to enter the low dielectric bilayer interior from a high dielectric aqueous solution, and showed that energies approaching 160 kJ mol^{-1} were required. Providing a hydrated defect (a channel) or surrounding the ion by an ononphore greatly reduced this energy. However, Born theory assumes an ionic radius, and therefore gives an absurd value when applied to bare hydrogen ions. Furthermore, Hauser *et al.* (1973) attempted to apply the

theory to ionic flux in small liposomes, and found that sodium flux was 1000-fold faster than predicted. It follows that the theory cannot be applied directly to quantitative descriptions of ionic conduction across lipid bilayer barriers, and must be modified in some way. The simplest possibility is that protons and other ions enter and move about in the bilayer as hydrated species in rare, transient defects. Therefore we will consider how water may be related to a general mechanism for ionic flux across bilayers (see Deamer and Bramhall, 1986) and specifically to proton flux mechanisms.

Proposed Mechanisms for Proton Conductance

To summarize the above introductory remarks, any mechanism proposed for proton conductance across lipid bilayers must account for two primary observations:

1. The proton permeability coefficient measured at pH 7, compared to other monovalent cations, is larger by approximately seven orders of magnitude.
2. Proton flux is relatively constant with pH.

There are no hypotheses yet available that account quantitatively for these observations, in the sense that a constant conductance or difference of six orders of magnitude can be shown to arise directly from accepted theories of conductance in ionic solutions. In fact, there are no bulk phase systems in which proton conductance deviates from other ionic conductance by such large factors. Therefore current approaches to this question are necessarily attempting to gain insight into the mechanism at a more empirical level.

Two explanations have been proposed for the proton permeability anomaly. Both assume that the bilayer itself is the primary barrier, and both involve a specific property of protons relative to other ions. The first hypothesis assumes that there is an unknown contaminant in lipid bilayers which acts to increase proton permeability. This was suggested by Gutknecht and Walter (1981) and is described in detailed elsewhere in this volume. We will use "protonophore" as a general term to describe such contaminants, with the understanding that the mechanism could differ from that of classical protonophores.

It is clear that proton conductance can be differentiated from that of other ions by protonophores, which are organic weak acids that partition into the membrane phase and discharge proton gradients by transporting protons as neutral species. Protonophores are powerful uncoupling agents, and in early work were shown to be capable of increasing current across planar lipid membranes by three orders of magnitude (Hopfer *et al.*, 1968; LeBlanc, 1971). Because the current is carried by the anionic form of the

protonophore (McLaughlin and Dilger, 1980), substances such as phloretin which alter membrane dipole potentials markedly affect the action of protonophores. It follows that phloretin offers a test of the protonophore hypothesis, as will be discussed later.

There is little doubt that the conditions under which lipid bilayers are prepared for study can introduce trace contaminants which may exert an unexpected effect on proton conductance. For instance, lipid hydrolysis could produce free fatty acids, and lipid oxidation could form a variety of oxidized species which might act as weak-acid protonophores. Cafiso and Hubbell (1983) first noted that proton permeability of liposomes is relatively low if care is taken to reduce possible hydrolysis and oxidation damage to minimal levels. Furthermore, proton permeability is relatively high in those systems which are most labile to such damage. For example, the permeability coefficient reported by Cafiso and Hubbell for egg phosphatidylcholine is about two orders of magnitude greater than for diphytanoylphosphatidylcholine.

The most extensive evidence has been obtained by Gutknecht (1987) who used several approaches to test the hypothesis in planar lipid membranes. First, if trace protonophores contribute to proton conductance, one would expect that bovine serum albumin (BSA), which strongly binds fatty acids and other hydrophobic contaminants, would reduce conductance. Gutknecht measured a tenfold decrease in conductance when BSA was present, to levels near the limit of sensitivity of the PLM method. Furthermore, if octanoic acid was added to saturate binding sites on the BSA, its effect on proton conductance was absent. Gutknecht also found that water permeability was not affected by the BSA, even though proton conductance was decreased. This suggested that water permeation and proton conductance may not be directly linked, at least in planar lipid membranes.

To determine whether fatty acids could affect bilayer permeability, Gutknecht added known amounts of phytanic acid, and observed that conductance increased about tenfold as fatty acid in the bilayer increased up to 0.2 mole fraction. This effect was reversed by BSA, as expected. Titrations of pH caused a change in conductance similar to that observed when titrations were done in the absence of added fatty acid, as though the pK of a protonophore was being titrated. Finally, the possibility that current was being carried by the anionic form of a protonophore was tested by addition of phloretin, which was found to reduce proton conductance approximately tenfold.

Critique of the Protonophore Hypothesis

Although these results are suggestive that trace contaminants may contribute to proton conductance in planar lipid membranes, they do not

provide a complete explanation of the proton permeability anomaly. The additions of BSA or fatty acid at most produce approximately tenfold changes in conductance in the planar lipid membrane system, and to achieve this effect substantial amounts (0.1–0.2 mole fraction) of fatty acid were added. Although a tenfold change represents a large fraction of the total proton current that can be measured in the planar lipid membrane system, it does not account for the six to seven orders of magnitude difference observed between proton and other cation permeabilities. Furthermore, the plot of conductance against pH (Gutknecht, 1984, 1987) differs considerably from that of known protonophores (Leblanc, 1971; see also Nagle, this volume). Finally, Gutknecht found that phloretin markedly reduced conductance across planar lipid membranes, which is consistent with the idea that anionic carriers are involved, but Perkins and Cafiso (1986) were unable to measure any effect of phloretin on proton flux in liposome systems.

Because of the clear effect of BSA on planar lipid membranes, it was important to determine whether similar effects could be observed in liposome systems. We have carried out such an experiment with phospholipid concentrations of 1 mg per ml and fatty acid free-BSA at 2 and 10 mg per ml. Assuming six high-energy hydrophobic binding sites per BSA molecule (Spector, 1967) the latter BSA concentration represented approximately one hydrophobic binding site per phospholipid present, presumably sufficient to remove traces of nonpolar contaminants. Proton flux was measured in egg PC : PA vesicles (90 : 10) by monitoring relaxation of pH gradients with a trapped dye (pyranine) method (Kano and Fendler, 1978; Clement and Gould, 1981). Vesicles were prepared by extrusion (Hope *et al.*, 1985) in 5 mM pH 8 buffer consisting of a mixture of zwitterionic buffers with approximately linear buffer capacity between 6 and 8. The extrusion method uses nitrogen pressure filtration to produce unilamellar liposomes of defined size ranges. The procedure can be completed in 20 minutes, and because no detergents or organic solvents are required, potential contamination is reduced to minimal levels. Potassium sulfate (0.2 M) was present so that osmotic or ionic strength changes were negligible when pH was varied, and the high potassium concentrations provided saturating amounts for valinomycin additions (1 μg per mg phospholipid) which were often included in an experiment to release proton-diffusion potentials. EDTA (1 mM) was present as a chelating agent. The pH outside was lowered by adding 0.1 N sulfuric acid in microliter quantities, producing pH gradients ranging from 0.3 to 1.3 pH units. the initial rate of decay was measured by decreased fluorescence of the dye, which was calibrated at the end of the experiment by back-titration with a known amount of 0.1 N NaOH. Net proton flux (moles $cm^{-2} s^{-1}$) was calculated as proton equivalents crossing the lipid bilayer area in response to the imposed gradient.

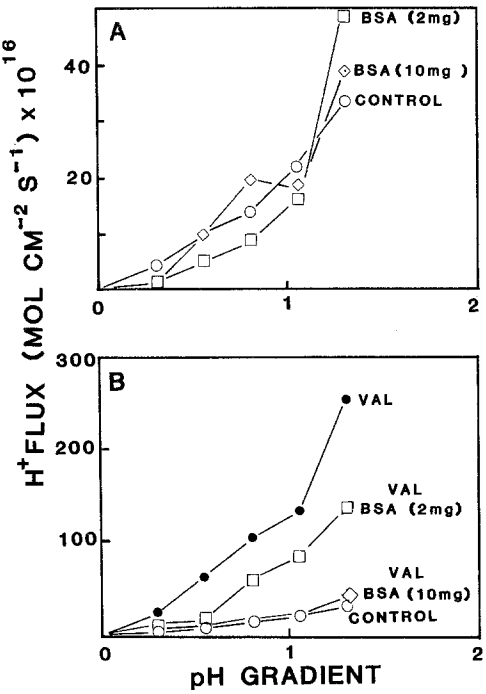

Fig. 1. Effects of BSA on proton flux in liposomes. Liposomes were prepared from egg PC : POPA (10 mg/ml, mole ratio 9 : 1) by nitrogen pressure extrusion through 0.2 μm polycarbonate filters (10 successive passes). The medium contained 0.2 M potassium sulfate, 0.5 mM pyranine, 1.0 mM EDTA, and 5 mM zwitterionic buffer (pH 8.0) containing ACES, MES, TES, and Tricine (Sigma Inc. St. Louis, Missouri) in equal concentrations to provide approximately linear buffer capacity between pH 6 and 8. The liposomes were passed through Sephadex G-50 gel in the same medium lacking pyranine, then adjusted to 5 mg/ml. The dye peak was also collected in 10 ml medium. Aliquots of the liposomes (0.2 ml) were added to 1.8 ml of the same medium in an Aminco Bowman fluorometer, and fluorescence was monitored at 430/515 ex/em. The volume ratio (V_i/V_o) was measured by comparing the fluorescence of encapsulated dye to that of the dye peak. This ratio was typically in the range of 2.0 μl per ml external medium in the cell. The pH was then reduced by additions of 0.1 M H^+ (10–50 μl sulfuric acid, producing pH gradients of 0.3 to 1.3 units), and the initial rate of fluorescence decrease was measured. At the end of an experiment, gramicidin (0.1 μg) was added to make the liposomes permeable to protons, and the fluorescence was back-titrated with 0.1 M NaOH in aliquots equal to the original acid additions. The final addition of NaOH (10 μl) was used to calibrate the initial rate of fluorescence change caused by the acid addition, and was multiplied by V_i/V_o to relate the fluorescence change to proton flux in the liposome volume. Bilayer area was taken to be 2500 cm^2 per mg phospholipid. In some experiments valinomycin (0.5 μg/mg PL) or BSA (2 and 10 mg/ml) were added to the cell before addition of liposomes. BSA had no effect on proton flux in the absence of valinomycin (A) but reduced flux in the presence of valinomycin (B).

Figure 1 shows our results. The presence of BSA had no effect on proton flux in the absence of valinomycin. Proton flux was increased in the presence of valinomycin, presumably because proton diffusion potentials limit flux as pH gradients decay across the lipid bilayer. This effect was more obvious at higher pH gradients where proton diffusion potentials became increasingly limiting to net flux. BSA at 2 mg/ml, ten times the concentration employed by Gutknecht, reduced proton flux by about half with valinomycin present, while BSA at 10 mg/ml reduced proton flux about fivefold. In control experiments, BSA additions had no effect on the marked increment in flux caused by gramicidin, a larger peptide which strongly binds to lipid bilayers and produces channels that conduct both protons and potassium (results not shown). Significantly, all of the plots of flux against pH gradient appeared to be superlinear, an observation which will be discussed later.

These results suggest that BSA does not have the same marked effect in liposomes as it seems to in PLM systems, at least in the absence of valinomycin. The reduction in proton conductance caused by BSA in the presence of valinomycin could be explained as binding of trace protonophores by BSA, but an alternative explanation is that valinomycin itself was interacting with BSA, since the flux was reduced only to control levels. (About 4000 BSA binding sites were available per valinomycin molecule.) A more precise understanding requires further work, but it is clear that the effect of BSA on proton flux cannot account for the proton permeability anomaly.

Hydrogen-Bonded Chains and Proton Translocation

The second hypothesis for proton permeation of bilayers also incorporates a unique property of protons, the capacity to undergo "hopping" along hydrogen-bonded chains. Onsager (1967) suggested that such conductance mechanisms may have biological relevance, and the term "proton wire" was coined by Nagle and Morowitz (1978), referring to such a mechanism in membranes. The term "hydrogen-bonded chain" (HBC) has been proposed as a general term by Nagle and Tristram-Nagle (1983).

When the first measurements of high proton permeability were reported (Nichols and Deamer, 1980), it was proposed that the permeability anomaly could arise if a fraction of membrane water was associated as hydrogen-bonded structures in transient defects. Such structures would clearly differentiate between protons and other ions, since only protons could undergo wirelike conductance along the chains. The convention tHBC (see Nagle, this volume) will be used here to differentiate transient chains of water molecules from the peptide strands originally proposed by Nagle and Morowitz.

One direct test of the tHBC hypothesis has been carried out so far, which follows from the prediction that, all other considerations being equal, an increase in the amount of intramembrane water should produce a proportional increase in proton conductance, but not necessarily of other cations which are unable to take advantage of tHBC. This experiment was carried out by Elamrani and Blume (1983) who measured proton permeability of liposomes above and below their phase transition. From past work, it is known that water permeation increases about two orders of magnitude when a bilayer undergoes a gel-to-fluid phase transition (Lawaczeck, 1979) but that permeability to ions such as sodium increases only severalfold at the phase transition and then decreases to near the original level (El-Mashak and Tsong, 1985). This suggests that sodium-conducting defects are relatively common near the phase transition, and less common above and below it. On the other hand, water-conducting defects become much more common in fluid than in solid lipid bilayers, and presumably water associated at tHBC increases as well, providing enhanced opportunities for proton flux.

Elamrani and Blume showed that proton permeability increased about two orders of magnitude as lipoomes maintaining pH gradients underwent a temperature-induced phase transition. This result is consistent with the tHBC hypothesis, but does not exclude the possibility that a protonophore contaminant happens to conduct protons at 100-fold greater rates in the fluid bilayer.

Critique of the tHBC Hypothesis

Although the tHBC hypothesis does not assume a universal protonophoric carrier in lipid bilayers, it does require something equally implausible, which is an ordering of water molecules into extended hydrogen-bonded chains in a nonpolar environment. Evidence related to this was presented by Conrad and Strauss (1985) who used infrared methods to determine the extent of hydrogen-bonded water in saturated decane and decene. Within the sensitivity of the method, no associated water could be detected. However, only a small fraction of membrane water needs to be associated to account for proton permeation, and ordering effects may occur in lipid bilayers which are not possible in isotropic bulk-phase hydrocarbons. Therefore, the Conrad and Strauss results do not critically exclude the tHBC hypothesis.

Perhaps the most problematic aspect of the tHBC model is that it does not arise from bulk-phase properties which can be described quantitatively in physicochemical terms, but instead invokes a process which may occur only in the microenvironment of the lipid bilayer. Therefore it has been difficult to find predictions of the model that can be tested experimentally.

Nonetheless, some progress has been made, particularly in proposing general mathematical models which discriminate among several kinds of tHBC conduction (Nagle, this volume). Another approach is to investigate proton conductance along the "water wire" which appears to exist in the gramicidin channel. The results can then be compared with proton conduction across lipid bilayers, as described below.

Models of HBC Proton Conductance

Although a mechanism involving associated water in lipid bilayers is conjectural, there is some agreement that protons move along hydrogen-bonded water chains in the gramicidin channel. Gramicidins are antibiotics produced by *Bacillus brevis*, with relative specificity in their action against Gram-positive bacteria. Gramicidin A is a major component of the mix ($\sim 70\%$) with the rest composed of closely related peptides. It is a pentadecapeptide, MW 1883 daltons, and has the sequence HCO-L-val-gly-L-ala-D-leu-L-ala-D-val-L-val-D-val-L-trp-D-leu-L-trp-D-leu-L-trp-D-leu-L-trp-$NHCH_2CH_2OH$. The alternating D and L amino acids are hydrophobic, permitting it to partition into the nonpolar portion of lipid bilayers. Hladky and Haydon (1971) first demonstrated that gramicidin A produced ion-conducting channels in planar lipid membranes, and Urry (1971) proposed that the molecular configuration was a π helix, with each helix having a relatively polar channel down its center. The channel is 0.2 nm in diameter, sufficient to accommodate a chain of water molecules (Fornili *et al.* 1985). A single gramicidin molecule is too small to span a lipid bilayer, and it is generally considered that the ion conducting channel is produced when two of the peptides transiently join head-to-head (Urry *et al.*, 1971; Bamberg *et al.*, 1976; Cornell *et al.*, 1987a, b).

Myers and Haydon (1972) found an unexpectedly high permeability of protons through the channel, and proposed a hopping mechanism along a continuous chain of water molecules. Later observations have been consistent with this suggestion. For instance, Levitt *et al.* (1978) measured streaming potentials as various cations passed through the channel down concentration gradients, and determined that about 12 water molecules were pushed through by ions such as sodium or potassium moving down 0.15 M concentration gradients. Streaming potentials could not be detected during proton conductance through the channel, consistent with the idea that a hopping mechanism was involved.

Proton flux through the gramicidin channels in small sonicated liposomes was first studied by Clement and Gould (1981) who found that only four to five channels per vesicle increased permeability to protons and counterions

by several orders of magnitude. Activation energies were found to be very high, ranging from 15 kcal mol^{-1} in phospholipid vesicles to 26 kcal mol^{-1} when 33 mol.% cholesterol was present. We have extended these studies to large unilamellar liposomes, and measured relative fluxes and permeabilities of protons and potassium ion under a variety of conditions. Assuming that the channel qualitatively resembles the tHBC proposed to account for proton flux across lipid bilayer membranes, we had two expectations. First, the proton to potassium permeabilities at neutral pH ranges should be different by several orders of magnitude. Second, the conductive properties of the channel should fit one of the three models proposed for proton transport by Nagle (this volume).

Proton flux was monitored by the pyranine method described earlier in Fig. 1, and potassium flux was measured under the same conditions by an ion-sensitive glass electrode, with choline replacing potassium in the external medium. Because potassium ions are unable to utilize hydrogen-bonded chains as a conductive mechanism, comparisons of proton/potassium flux and permeabilities provided a useful tool for studying permeation mechanisms. Gramicidin A′ (Sigma Inc., St. Louis, Missouri) was used without further purification. In some experiments, deuterium oxide was substituted for water to look for possible isotope effects that would provide further information about proton flux mechanisms.

Gramicidin Affects Liposome Structure Only at High Peptide-to-Lipid Ratios

In preliminary experiments, the effect of gramicidin additions on the structure of liposomes was monitored by freeze-fracture electron microscopy. Some typical results are shown in Fig. 2. At gramicidin/phospholipid mole ratios of 1 : 100, far greater than those causing increments in ion conductance ($1 : 10^4$), freeze-fracture images showed no obvious differences from control liposomes. This suggested that gramicidin molecules embedded in bilayers are below the resolution of the freeze-fracture technique, which is not surprising, in that we would only expect to visualize a small peptide if it aggregated in the bilayer or produced a defect involving hundreds of lipid molecules.

At higher concentrations of gramicidin (mole ratio 1 : 12.5), large vesicles dominated freeze-fracture images, indicating that gramicidin causes membrane fusion at sufficiently high ratios of peptide to lipid. Killian *et al.* (1985) showed that gramicidin induces hexagonal phases in phospholipids when present at mole ratios exceeding 0.15, so a fusigenic effect was not unexpected.

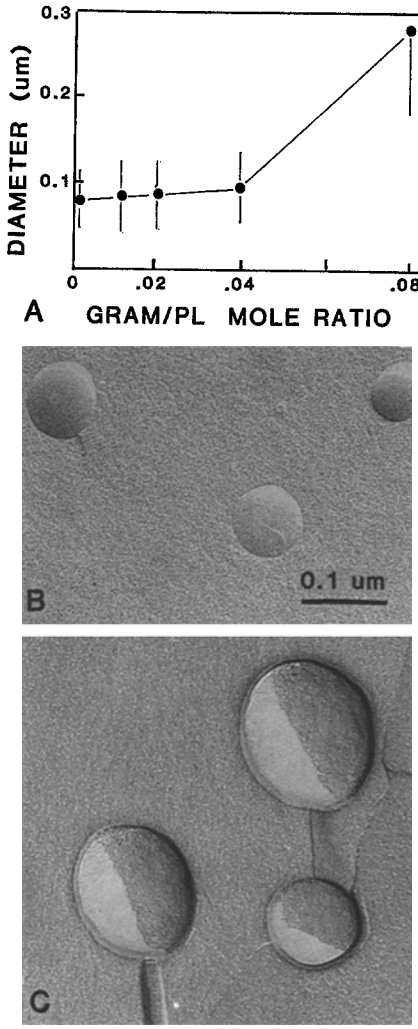

Fig. 2. Effect of gramicidin on liposome structure visualized by the freeze-fracture method. Mean diameters of 50 randomly chosen vesicles are shown for five different gramicidin/phospholipid ratios in the graph (A). At lower peptide/phospholipid ratios (B) liposomes were indistinguishable from controls. At higher ratios, vesicle diameters were markedly increased (C). There was considerable aggregation and heterogeneity in such preparations, with some hexagonal phase lipid present (not shown). Although fracture faces appeared rougher than control liposomes, individual particles were not observed. These results show that gramicidin causes physical changes in lipid bilayer properties, but only at ratios three orders of magnitude higher than those producing permeability increments.

Gramicidin Channels Are Highly Selective for Protons

The gramicidin channel provided a test of the concept that hydrogen-bonded chains of water can be selective for protons diffusing down concentration gradients. If the permeability anomaly in bilayers results from transient hydrogen-bonded chains of water, a similar result should be observed with flux through the gramicidin channel. To make this measurement, gramicidin was titrated into liposomes maintaining either pH gradients or potassium concentration gradients, and the increments in flux were measured. The incremental flux was then divided by the number of channels (gramicidin dimers) to obtain the unit flux (ions per channel per second), and it was found that unit flux of potassium was about 100 times that of protons under the experimental conditions used (Fig. 3). However, the proton flux was driven by concentration gradients in the 0.1 μM range, while the potassium flux was driven by 0.4 M gradients. When unit permeability coefficients were calculated by dividing the flux by the concentration gradients, the gramicidin channel was found to be 5×10^4 times more permeable to protons than to potassium.

It is interesting to compare this value with results reported by Myers and Haydon (1972) who measured H^+/Na^+ permeability ratios ranging from approximately 50 to over 300, depending on experimental conditions. However, their measurements were carried out in 0.01 to 0.1 M H^+, and Gutknecht (1984) has clearly shown that the permeability anomaly is greatly reduced at lower pH ranges because proton conductance is relatively independent of pH. In future work it will be interesting to determine whether proton conductance through the gramicidin channel is also relatively independent of pH.

It was also possible to use the gramicidin results to calculate the fraction of intramembrane water associated as tHBC needed to account for the measured proton conduction. This calculation can at best provide order-of-magnitude estimates, since it assumes a water concentration in lipid bilayers which is based on bulk-phase values, and also assumes that the motionally restricted water in the gramicidin channel resembles hypothetical tHBC in fluid lipid bilayers. However, even a rough estimate may provide insight, in that an unreasonable requirement for associated water would argue against the tHBC mechanism.

From the data given in Fig. 3, it can be calculated that one gramicidin channel or one equivalent tHBC per 10^5 phospholipid molecules could transport sufficient protons to account for the measured flux down a gradient of 1 pH unit with valinomycin present. It can be estimated from water solubility in bulk-phase hydrocarbons that about 300 water molecules are present per 10^5 phospholipids in a lipid bilayer (Biegel and Gould, 1982).

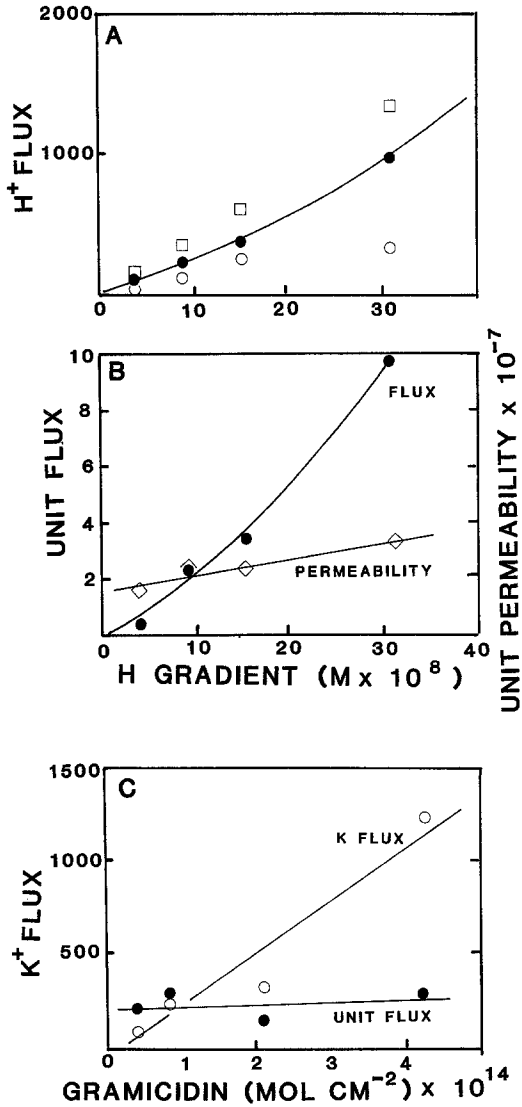

Fig. 3. Relative permeability of gramicidin channels to protons and potassium ions. Proton flux (mol $cm^{-2} s^{-1} \times 10^{16}$) was measured as described in the text and Fig. 1, and potassium flux (mol $cm^{-2} s^{-1} \times 10^{14}$) was measured by a potassium-sensitive glass electrode, with choline replacing potassium in the external medium. Valinomycin (0.5 μg/mg lipid) was present during proton flux measurements to prevent proton diffusion potentials from interfering. Gramicidin concentration was 100 ng/mg phospholipid (1.05×10^{-14} mols channel cm^{-2} bilayer) for proton flux measurements (egg PC : POPA liposomes, 9 : 1, 1 mg total lipid). Gramicidin was varied from 0 to 100 ng in potassium flux measurements (5 mg total lipid), and its concentration in the figures is given as mols channel $cm^{-2} \times 10^{14}$. To measure proton flux, gramicidin concentration

Since a transmembrane water chain requires about 20 molecules, 20 in 300 waters, or 7%, would need to be present as tHBC resembling the gramicidin channel to account for the proton permeability anomaly. This figure is surprisingly large, but probably represents an upper limit, because the calculation assumes that all the added gramicidin forms channels, and all channels are open. In fact, the channel is itself transient, and depends on the formation of gramicidin dimers with lifetimes ranging from less than a second to several hundred seconds, depending on the environment (Haydon, 1975).

Effect of Deuterium Oxide on Proton and Potassium Conductance through the Gramicidin Channel

The concept of proton hopping arose from measurements of conductance in water and ice, in which proton conductance is much greater than can be explained by simple diffusion. It is worth asking whether some of the information available from studies of ice may be applicable to the concept of tHBC in bilayers. The conductive properties of ice have been the focus of considerable research interest (Eigen and DeMaeyer, 1958; Bullemer *et al.*, 1968; von Hippel *et al.*, 1971). Measurements of current are complicated by surface conductance and polycrystalline structure of ice, and the resulting variance in reported values has hindered development of a comprehensive theory. However, there is agreement that at least two types of defects must be involved in the conduction mechanism: orientational defects (Bjerrum, 1951) and ionic defects. Conductance of current involves hopping of ionic defects present as H_3O^+ and OH^-, and the passage of an ionic defect must be followed by reorientation of water molecules to accommodate the arrival of a new hopping defect. Proton flux along tHBC in membranes can be understood in similar terms (Nagle, this volume).

If proton flux across bilayers does proceed by a hopping mechanism along associated water, one might expect to find isotope effects if D_2O is substituted for H_2O. The physical properties of deuterium oxide ice

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was kept constant and pH gradients were varied from 0.3 to 1.3 pH units across liposome membranes (A). The open circles show control values, and open squares show proton flux with gramicidin present. The closed circles and line give the difference, which was taken to represent proton flux through gramicidin channels. Potassium flux was measured with a gradient of 0.4 M potassium ion, and gramicidin was varied as shown in (C). From the flux increments caused by gramicidin additions, unit flux could be calculated as ions per channel per second. This ranged from 1 to 10 protons per second as the pH gradient increased from 0.3 to 1.3 pH units (B) and was approximately 230 potassium ions per channel per second (C) The unit permeability coefficients were then calculated as unit flux/concentration, giving values of 3×10^7 for protons and 585 for potassium ions, with a H^+/K^+ permeability ratio of 5×10^4.

resemble water ice in most respects, but there are small differences in the bond energies of D_2O which produce measurable effects. Probably the most striking is that the conductance of deuterium oxide ice is approximately two orders of magnitude lower than water ice (Eigen, 1964). There is no simple way to predict what effects one might expect in comparing physical properties of water organized in ice with that in tHBC, but if differences in proton conductance could be demonstrated in D_2O, it would justify further investigation. Perkins and Cafiso (1986) have carried out initial studies with liposomes, and we have extended their approach to the gramicidin channel as well. Gradients of about 1 pH unit were produced across liposome membranes in buffer solutions with varying ratios of H_2O to D_2O, and in the presence and absence of valinomycin or gramicidin. The decay of the pH gradient was monitored by the pyranine method described earlier. Potassium ion gradients of 0.4 M were produced in the same liposome system, and efflux was measured with a potassium electrode.

Figure 4 shows averaged results for five separate experiments. In the absence of gramicidin, we could find no significant differences between proton flux measured in water and deuterium oxide, within the noise of data points from multiple experiments. However, in the presence of enough gramicidin to increase proton conductance about tenfold, there was an easily measured linear decrease in proton flux as the buffer was varied from 100% H_2O to 90% D_2O. Potassium flux was unaffected by D_2O, in the presence or absence of gramicidin.

The lack of a D_2O effect on proton flux in liposomes confirms the observations of Perkins and Cafiso. However, the noise in the data was substantial, and could easily mask a modest change in flux. In fact, the mean flux decreased by about half in the experiment described by Perkins and Cafiso (1986), but was not statistically significant.

The proton flux in the presence of gramicidin was greater, and the noise less, so that a decreased flux could more readily be observed. The fact that potassium flux was not affected increases our confidence in the result. This represents the first demonstration of an isotope effect on proton translocation along a hydrogen-bonded chain of water in membranes. The origin of the effect is not clear, and may not arise from the same causes as the decreased conductance observed in D_2O ice. However, the result agrees with earlier suggestions that proton and potassium flux through the gramicidin channel occurs by different mechanisms. Furthermore, it provides a potential tool to determine whether functional proton transport occurs along tHBC in membrane proteins such as bacteriorhodopsin and the F_0 subunit of coupling factors.

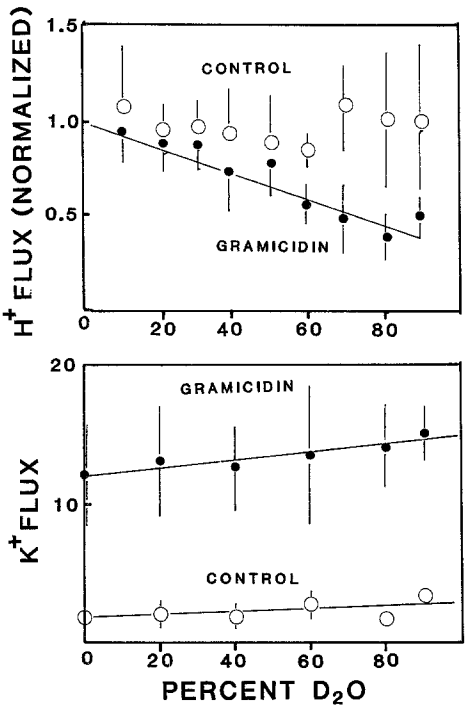

Fig. 4. Effect of D_2O on proton and potassium flux. Proton flux was monitored by the pyranine method described in the text and Fig. 1, and potassium flux was measured with a glass electrode as described in Fig. 3. Because of approximately 2-fold variation between liposome preparations, the proton flux was normalized, with values in 100% H_2O taken as 1.0. Means of four runs + S.D. are shown, and lines are drawn by standard linear regression analysis. Potassium flux is given as mol $cm^{-2} \times 10^{14}$. The results show a clear trend to lower proton flux through the gramicidin channel as D_2O increased, but no effect in the absence of gramicidin. Potassium flux was not affected by D_2O.

What is the Shape of the Proton Conductance Curve as ΔpH Is Varied?

This question arises from three models of proton flux along hydrogen-bonded chains which are described elsewhere in this volume by Nagle. The first two models consider tHBC with lifetimes permitting multiple proton or single proton translocation (models A and B) and the third model considers partial tHBC which permit translocation of proton equivalents (by recombination of H_3O^+ and OH^-) only when two chains touch across the bilayer. Each model predicts a characteristic curve when proton flux is plotted against

the magnitude of the pH gradient driving flux, the curves being sublinear for models A and B, and superlinear for model C.

We have undertaken to test these models, using the pyranine dye system described earlier, in the presence and absence of valinomycin and gramicidin. Valinomycin minimized possible effects of proton diffusion potentials, and gramicidin provided a model tHBC. Figure 5 shows a summary of the results. Proton conductance plotted against pH appeared to be superlinear, both in the presence and absence of valinomycin (Fig. 5A), and the data points fell near a theoretical curve plotted from model C. Superlinearity was also observed when sufficient gramicidin was added to increase conductance by approximately tenfold (Fig. 5B). This is significant, in that gramicidin would conduct protons only when two conditions are met: a gramicidin dimer must form in the correct head-to-head configuration, and water molecules in the channel must associate into a transient hydrogen-bonded chain. Nagle's model C predicts that superlinearity would result if the proton conductance mechanism involves transient associated water structures which touch across the bilayer and discharge proton–hydroxide pairs. Gramicidin might therefore provide a test of the theory, but it is first necessary to known the channel lifetime relative to the unit flux of protons. An initial estimate of unit flux at pH 7 is 3 protons per channel per second (Fig. 3). If the channel lifetime is in the range of a second or longer, the proton flux would be described by model A (see Nagle, this volume) and should produce sublinear plots of flux against pH gradients. The superlinear plots observed here are better described by model C, and it follows that channel lifetime in phospholipid bilayers will be less than a second. This question remains to be resolved.

An important test of the above results is to repeat the experiments under conditions in which protons are not being translocated by hypothetical tHBC, for instance, when most of the proton current is carried by a protonophore. These experiments are in progress, and some preliminary results are shown in Fig. 5C, in which a protonophore was added together with valinomycin so that flux was at least two orders of magnitude greater than in the absence of the ionophore and protonophore. With most of the proton current being carried by FCCP, the plot of flux against pH gradient was linear.

Although the results described here are consistent with model C of Nagle, they cannot be considered conclusive. For instance, Kell and Morris (1980), using soya-bean phosphatidylcholine vesicles, reported that proton flux increased linearly with pH gradients up to 4 units. O'Shea *et al.* (1984) confirmed this observation, but observed superlinearity when proton flux was driven by potassium diffusion potentials from 20 to 160 mV. The plots became linear when a protonophore (CCCP) was added, in agreement with the results described here.

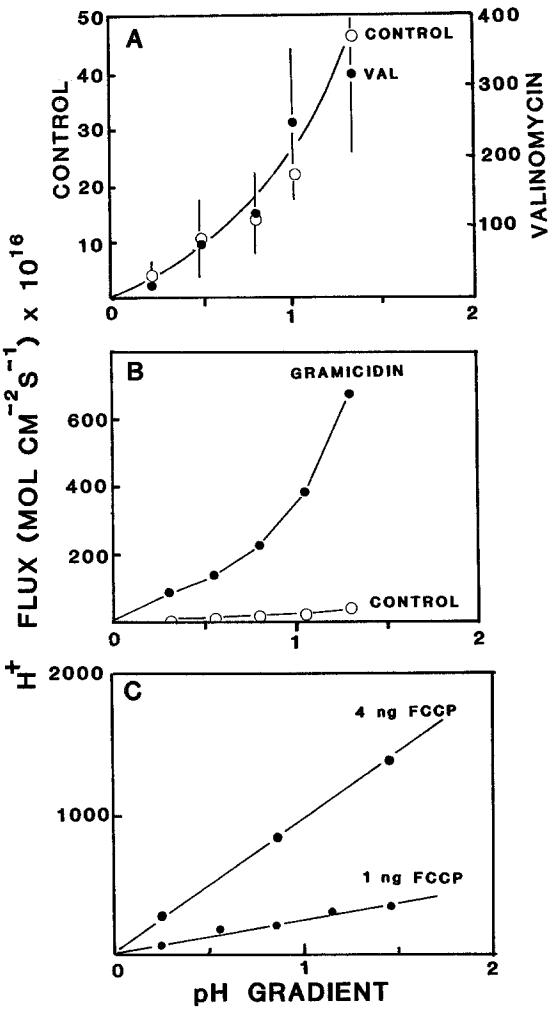

Fig. 5. Relation between proton flux and pH gradient in liposomes. Proton flux was measured as described in Fig. 1. (A) The means of four separate experiments, for controls and valinomycin additions (0.5 μg/mg PL). Standard deviations are given for the valinomycin data. The line is a theoretical curve from model C plotted from the equation $J = J_0(\sinh[\beta\delta])$, where $\beta = 1/kT$ and $\delta = 2.3kT\Delta pH$. (See Nagle, this volume.) (B) An experiment in which gramicidin (0.1 μg/mg PL) was present, and comparison of the plot of flux vs pH gradient against a control experiment using the same liposome preparation. (C) Internal buffer was increased to 50 mM in order to measure higher flux rates, and valinomycin (0.5 μg/mg PL) and two concentrations of FCCP (1 and 4 ng/mg PL) were present. The plots of flux against pH gradient appear linear.

Despite the discrepancies which remain to be resolved, it is clear that some new approaches to a difficult problem have been found. There is an increasing consensus about the kinds of questions which will further our understanding of proton-hydroxide flux across bilayer membranes. A particularly exciting possibility is that the mechanism may not be limited to processes occurring in bilayers, but will also be relevant to biological membrane function.

Acknowledgments

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