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# Proton / hydroxide conductance through phospholipid bilayer membranes: effects of phytanic acid

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**Mechanisms of proton/hydroxide conductance ($G_{H/OH}$) were investigated in planar (Mueller-Rudin) bilayer membranes made from decane solutions of phospholipids or phospholipids plus phytanic acid (a 20-carbon, branched chain fatty acid). At neutral pH, membranes made from diphytanoylphosphatidylcholine or bacterial phosphatidylethanolamine had $G_{H/OH}$ values in the range of $(2–5) \cdot 10^{-9}$ S · cm$^{-2}$, corresponding to $H^+/OH^-$ 'net' permeabilities of about $(0.4–1.0) \cdot 10^{-5}$ cm · s$^{-1}$. $G_{H/OH}$ was inhibited by serum albumin, phloretin, glycerol and low pH, but was increased by chlorodecane and voltage > 80 mV. Water permeability and $G_{H/OH}$ were not correlated, suggesting that water and $H^+/OH^-$ cross the membrane by separate pathways. Addition of phytanic acid to the phospholipids caused an increase in $G_{H/OH}$ which was proportional to the first power of the phytanic acid concentration. In membranes containing phytanic acid, $G_{H/OH}$ was inhibited by albumin, phloretin, glycerol and low pH, but was increased by chlorodecane and voltages > 80 mV. The results suggest that phytanic acid acts as a simple ($A^-$ type) proton carrier. The qualitative similarities between the behavior of $G_{H/OH}$ in unmodified and phytanic-acid containing membranes suggest that phospholipids may contain weakly acidic contaminants which cause most of $G_{H/OH}$ at pH > 4. However, there is also a significant background (pH independent) $G_{H/OH}$ which may be due to hydrogen-bonded water chains. The ability of phytanic acid to act as a proton carrier may help to explain the toxicity of phytanic acid in Refsum's disease, a metabolic disorder in which phytanic acid accumulates to high levels in plasma, cells and tissues.**

## Introduction

Proton/hydroxide ($H^+/OH^-$) permeability of phospholipid bilayers at physiological pH is several orders of magnitude higher than alkali or halide ion permeability [1–13], but the mechanism of $H^+/OH^-$ permeability is unknown (for recent reviews, see Refs. 14 and 15). The primary purpose of this study was to investigate the mechanism(s) of $H^+/OH^-$ permeability and conductance in phospholipid bilayer membranes. Our initial objective was to identify conditions which either enhance or inhibit $G_{H/OH}$. Our second objective was to compare the behavior of $G_{H/OH}$ in unmodified bilayers and in bilayers containing phytanic acid, a 20-carbon, branched-chain fatty acid.

Abbreviations: CCCP, carbonylcyanide *m*-chlorophenylhydrazone; FCCP, carbonylcyanide *p*-trifluoromethoxyphenylhydrazone; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine.

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We chose phytanic acid for two reasons. First, we suspected that part of $G_{H/OH}$ might be due to traces of weakly acidic contaminants in the phospholipids. Phytanic acid, for example, is a possible contaminant in diphytanoylphosphatidylcholine. Second, phytanic acid accumulates to high levels in the plasma, cells and tissues of individuals with Refsum's disease (phytanic acid storage disease) [16,17]. Thus, we wished to see whether the effects of phytanic acid on lipid bilayers might provide any insights concerning the mechanisms of phytanic acid toxicity in Refsum's disease and related metabolic disorders.

We found that phytanic acid increases $G_{H/OH}$ in lipid bilayers, and the increase is proportional to the first power of the phytanic acid concentration. Furthermore, $G_{H/OH}$ increases with pH, membrane voltage and chlorodecane. Inhibitors of $G_{H/OH}$ include low pH, serum albumin, phloretin and glycerol. The behavior of $G_{H/OH}$ in unmodified bilayers is qualitatively similar to the behavior of $G_{H/OH}$ in phytanic acid containing bilayers, i.e., pH dependence, voltage sensitivity, enhancement by chlorodecane and inhibition by serum albumin, phloretin and glycerol. Thus, $G_{H/OH}$ in unmodified bilayers may be due in part to the presence of weakly acidic contaminants in the phospholipids. However, there is also a significant 'background' (pH independent) $G_{H/OH}$ which may be due to hydrogen-bonded chains of water molecules. The ability of phytanic acid to increase $G_{H/OH}$ in bilayers suggests a possible mechanism for phytanic acid toxicity in Refsum's disease. A preliminary account of this work has been published [18].

## Methods and Materials

Planar (Mueller-Rudin) [19] membranes were formed from bacterial phosphatidylethanolamine (PE) or diphytanoylphosphatidylcholine (PC) or egg PC plus cholesterol (1 : 1, mol ratio) in *n*-decane. In some experiments, phytanic acid and/or chlorodecane were included in the lipid solution.

The membranes were formed on a 2.0 $mm^2$ hole in a polyethylene partition mounted in a Plexiglas chamber. The chamber was designed so that one side of the membrane could be perfused continuously while tracer fluxes and electrical properties were measured [11,20].

The method of measuring $H^+/OH^-$ conductance and permeability is described elsewhere [11]. Briefly, the membranes were exposed to small (0.3–0.8 unit) pH gradients produced by various mixtures of weakly acidic and weakly basic buffers. In most experiments the concentrations were adjusted so that the front and rear solutions contained similar concentrations of all ions except $H^+$ and $OH^-$. Small (< 1.0 mM) differences between the buffer ion concentrations, $[A^-]$ and $[BH^+]$, were neglected because they contributed less than 1 mV to the $A^-$ and $BH^+$ equilibrium potentials. An example is shown in Fig. 1.

$H^+/OH^-$ diffusion potentials were measured by calomel-KCl electrodes and a high-impedance electrometer. $H^+/OH^-$ transference numbers ($T_{H/OH}$) were calculated from the relation, $T_{H/OH} = V_m/E_{H/OH}$, where $V_m$ is the diffusion potential, and $E_{H/OH}$ is the $H^+/OH^-$ equilibrium potential, calculated by the Nernst equation. $H^+/OH^-$ conductances ($G_{H/OH}$) were calculated from the relation, $G_{H/OH} = T_{H/OH}G_m$, where $G_m$ is the total specific membrane conductance in $S \cdot cm^{-2}$.

The method of measuring tracer fluxes and permeabilities is described elsewhere [20]. Briefly, after the membrane had thinned, 10 $\mu$Ci of tritiated water ($^3H_2O$) was injected into the rear compartment (1.1 ml), and the rate of tracer appearance in the front compartment was measured

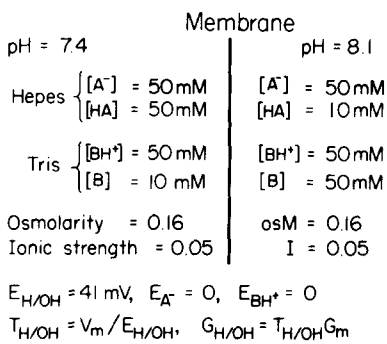


Fig. 1. An example of ionically and osmotically balanced buffer mixtures for producing a transmembrane $H^+/OH^-$ gradient. $A^-$ represents the Hepes anion, and HA represents the zwitterionic species ($pK = 7.4$). B represents the Tris free base, and $BH^+$ is the Tris cation ($pK = 8.1$). Also shown are the equilibrium potentials for $H^+/OH^-$, $A^-$ and $BH^+$, and the equations for calculating $T_{H/OH}$ and $G_{H/OH}$.

by continuous perfusion and collection of samples at 3-min intervals. The rear compartment was covered by a Teflon plug to prevent distillation of tracer into the front compartment. Both front and rear solutions were stirred magnetically. Temperature was $24 \pm 1°C$.

The unstirred layer thickness ($d$) in our system is $119 \pm 20$ $\mu$m [20–22], similar to the value obtained by others [23]. From this value the unstirred layer permeability to $^3H_2O$ was calculated, i.e., $P^{ul} = D/d$, where $D$ is the $^3H_2O$ diffusion coefficient in water ($2.4 \cdot 10^{-5}$ cm$^2 \cdot$ s$^{-1}$ at 25 °C). Then the membrane permeability ($P$) was calculated from the relation, $1/P = 1/P^{tot} - 1/P^{ul}$, where $P^{tot}$ is the total permeability, i.e., the permeability of the membrane plus unstirred layers. In this study the unstirred layer correction ranged from 32 to 39%.

Phospholipids were obtained from Avanti (Birmingham, AL). Decane (99.9%) was obtained from Wiley Organics (Columbus, OH), and 1-chlorodecane (95%) was obtained from Aldrich (Milwaukee, WI). Phytanic acid (3,7,11,15-tetramethylhexadecanoic acid) (99 + %) was obtained from Foxboro/Analabs (North Haven, CT). Buffers were obtained from Research Organics (Cleveland, OH). Tritiated water was obtained from ICN (Irvine, CA). Bovine serum albumin, cholesterol, phloretin and tetraphenylarsonium chloride were obtained from Sigma (St. Louis, MO).

The decane and chlorodecane were passed through an alumina column to remove polar impurities. Water was deionized and then doubly distilled. Before each experiment the membrane support was boiled for at least 4 h in several large volumes of ethanol plus NaOH (0.1 M).

## Results

### *$H^+/OH^-$ selectivity and conductance*

Fig. 2 shows diffusion potentials produced by $H^+/OH^-$ gradients across three types of membranes over the pH range 7.1 to 8.4. The pH gradients were produced by Hepes-Tris mixtures such as those shown in Fig. 1. In some experiments, KCl (10 mM) was added to 'buffer' any leakage from the KCl-calomel electrodes. The addition of KCl had little effect on $H^+/OH^-$ selectivity. $H^+/OH^-$ selectivity increased slightly at high pH (> 8) and decreased at low pH (< 6). At very low pH (1–3), all types of membranes were relatively nonselective, i.e., $T_{H/OH} = 0.4$ to 0.6.

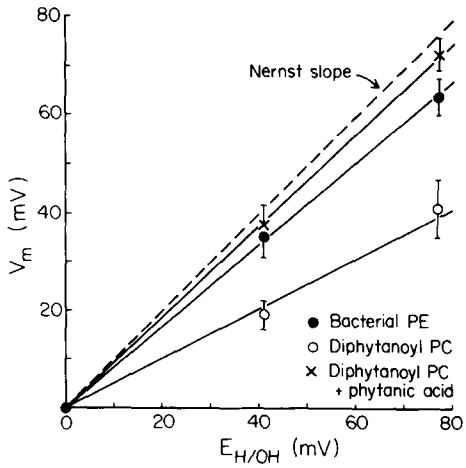


Fig. 2. Diffusion potentials produced by $H^+/OH^-$ gradients across three types of lipid bilayer membranes over the pH range of 7.1–8.4. The pH gradients were produced by Hepes-Tris mixtures such as those shown in Fig. 1. $E_{H/OH}$ is the $H^+/OH^-$ equilibrium potential. The dashed line has a slope of 1.0. Error bars are standard deviations of measurements on at least three membranes.

Fig. 3 shows $H^+/OH^-$ conductance ($G_{H/OH}$)

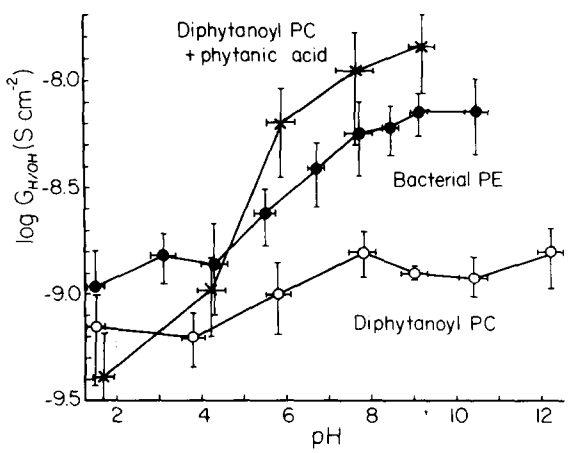


Fig. 3. Relation between $H^+/OH^-$ conductance and pH in three types of lipid bilayer membranes. Aqueous solutions were buffered as described in Fig. 1 and Ref. 11. Horizontal bars indicate the range of pH for each point, and vertical bars indicate standard deviations. The phytanic acid concentration was 8 mM (lipid mole fraction = 0.20, or 10% w/w).

as a function of pH in three types of membranes. In both bacterial PE and diphytanoylPC bilayers, $G_{H/OH}$ was low and relatively constant at pH < 4. $G_{H/OH}$ increased over the pH range of 4 to 8, and was fairly constant at pH > 8. The values of $G_{H/OH}$ at very high and very low pH should be viewed with caution, because pH extremes increase the rate of breakdown of phospholipids [24,25]. However, we found no evidence of phospholipid breakdown during our experiments. For example, at pH 12 the $G_{H/OH}$ of one diphytanoylPC membrane was constant ($1.8 \pm 0.3$ nS · cm$^{-2}$) for 1 h. At pH 10 the $G_{H/OH}$ of one bacterial PE membrane was constant ($9 \pm 1$ nS · cm$^{-2}$) for 1.5 h. Stable black films of bacterial PE would not form at pH > 11.

Membranes containing phytanic acid also showed a pH-dependent $G_{H/OH}$ (Fig. 3). At pH < 4, $G_{H/OH}$ was low and roughly similar to the values for PC and PE bilayers. Over the pH range of 4 to 8, $G_{H/OH}$ increased and appeared to saturate at pH > 8. Unfortunately, at pH > 9.5 the phytanic acid containing bilayers were mechanically unstable. However, at all other pH values, phytanic acid containing membranes were mechanically stable and showed constant $G_{H/OH}$ values for up to 1 h. Qualitatively similar results were obtained in preliminary experiments with palmitic and oleic acids over the pH range of 5.9–8.4. Decanoic acid, however, produced only transient (< 15 min) increases in $G_{H/OH}$.

Part of the statistical variation in $G_{H/OH}$ is due to a 2-fold variation among different batches of lipids. Also, the precision of the $G_{H/OH}$ measurements decreased at low $G_{H/OH}$ (< 1.5 nS · cm$^{-2}$). The lower limit of measurement in our system is about 0.1 nS · cm$^{-2}$.

*Effects of phytanic acid and chlorodecane on $G_{H/OH}$*

Fig. 4 shows the relation between $G_{H/OH}$ and phytanic acid concentration in bilayers made from diphytanoylPC in decane or 1-chlorodecane (30% v/v) in decane. In both types of membranes, $G_{H/OH}$ was proportional to the first power of the phytanic acid concentration. However, $G_{H/OH}$ was 8–10-fold higher in the chlorodecane containing membranes.

We used chlorodecane for two reasons. First, chlorodecane increases ionic (e.g., $SCN^-$ and

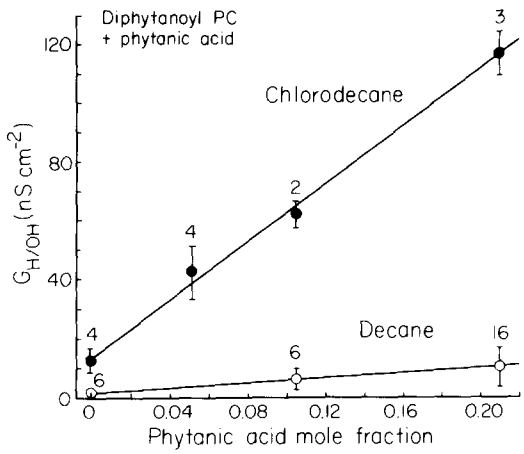


Fig. 4. Relation between $H^+/OH^-$ conductance and phytanic acid mole fraction in bilayers formed from diphytanoylPC (30 mM) in decane or 1-chlorodecane (30% v/v) plus decane. The pH gradient was 7.4–8.1. Also shown are standard deviations and the numbers of membranes.

$ClO_4^-$) permeability by increasing the dielectric constant of the membrane interior [26]. Thus, we thought that chlorodecane might be useful as an 'amplifier' to study low $H^+/OH^-$ conductance mechanisms in bilayers. Second, with respect to passive ionic permeabilities, chlorodecane bilayers may be good models for some biological membranes, e.g., mitochondria [26,27], which contain large amounts of integral membrane proteins.

We found chlorodecane-phospholipid membranes to be rather unstable, especially at low ionic strengths. However, a concentration of 30% (v/v) chlorodecane in decane produced the desired enhancement of $G_{H/OH}$ without loss of stability. The enhancement of $G_{H/OH}$ by 30% chlorodecane ranged from about 3–10-fold in several types of membranes (Table I and unpublished data). Increasing the chlorodecane concentration from 30% to 100% increased the conductances almost one order of magnitude above the values shown in Table 1.

*Current-voltage relationships*

Several studies have shown $H^+/OH^-$ current-voltage curves to be nearly linear at low voltage (< 80 mV) but superlinear at higher voltages [9–11]. One possible explanation for the nonlinearity is that the charge carrier crosses a

TABLE I

EFFECTS OF PHYTANIC ACID, 1-CHLORODECANE, SERUM ALBUMIN, PHLORETIN AND GLYCEROL ON $H^+/OH^-$ CONDUCTANCE [a]

| Lipids | $G_{H/OH}$ ($nS \cdot cm^{-2}$) | | | | |
|---|---|---|---|---|---|
| | Control | 1-Chlorodecane [b] (30% v/v) | Serum albumin [c] (0.1–0.2 $mg \cdot ml^{-1}$) | Phloretin [d] (0.1 mM) | Glycerol [e] (9.6 M) |
| Bacterial PE (26 mM) | 5.6 ± 1.8 (11) | 18.1 ± 5.2 (3) | 0.26 ± 0.11 (5) | < 0.2 (2) | 0.60 ± 0.23 (7) |
| DiphytanoylPC (30 mM) | 1.6 ± 0.4 (8) | 12.8 ± 4.7 (8) | 0.25 ± 0.08 (4) | < 0.2 (3) | 0.18 ± 0.08 (3) |
| DiphytanoylPC (30 mM) + phytanic acid (8 mM) [b] | 10.7 ± 4.9 (16) | 117 ± 7 (3) | 0.29 ± 0.12 (4) | < 0.4 (4) | 1.0 ± 0.18 (3) |

[a] Results are quoted as means ± S.D. (number of membranes). The pH gradient was 7.4–8.1. For comparison with the permeability coefficients calculated by others, a $G_{H/OH}$ of 1.0 $nS \cdot cm^{-2}$ corresponds to a $P_{H/OH}$ of $2.7 \cdot 10^{-6}$ $cm \cdot s^{-1}$ at pH 7.

[b] Chlorodecane and phytanic acid were added to the membrane forming solutions. The other agents were added to the aqueous phases.

[c] Albumin was fatty acid free ( < 0.005%) (Sigma No. A 0281).

[d] Precision was lower in the phloretin experiments because of slow transients in the diffusion potentials (see Ref. 38 for a discussion of this problem). The active species of phloretin is the protonated (nonionic) form, and the intrinsic p$K$ is 7.3 [38]. However, in PC bilayers phloretin remains primarily in the protonated form to at least pH 8.0 [12].

[e] In the glycerol experiments the pH range was 8.2 to 9.2. The concentration by weight was 75%. See Ref. 11 for additional data.

trapezoidal potential energy barrier in the membrane [28–30]. If so, then the normalized membrane conductance can be described by the relation

$$G_V/G_{40} = b \sinh(u/2)/\sinh(bu/2) \quad (1)$$

where $G_V/G_{40}$ is the ratio of the conductance at voltage, $V$, to the conductance at 40 mV, $b$ is the fraction of the membrane spanned by the minor base of the trapezoid, $u = FV/RT$, and $R$, $T$ and $F$ have their usual meanings.

Fig. 5 shows normalized $G$–$V$ data for several types of membranes, along with theoretical $G$–$V$ curves for three different values of $b$. The data are reasonably well fit by a value of $b = 0.6$–$0.7$, similar to some previous studies of $H^+/OH^-$ conductance through several types of lipid bilayers and mitochondrial membranes [10,32,33].

A difficulty in the interpretation of the $G$–$V$ data is that we are not certain that the increased conductance at high voltage is due solely to $H^+/OH^-$. In most membranes, $T_{H/OH} < 1.0$, especially at pH < 7. Furthermore, membranes often ruptured at voltages ranging from 120 to 160 mV and always ruptured near 200 mV. Thus, these data must be interpreted with caution.

*Effects of serum albumin, phloretin and glycerol on $G_{H/OH}$*

Serum albumin is well known for its ability to bind amphiphilic molecules and ions, e.g., fatty acids [34–37]. Figs. 6 and 7 show that serum albumin is also an inhibitor of $H^+/OH^-$ conductance in phospholipid bilayers. Qualitatively similar results were obtained in membranes made from PE, PC, PC plus phytanic acid, PC plus myristic or oleic acids (data not shown), and egg PC plus cholesterol (Fig. 8). In each case, albumin reduced $G_{H/OH}$ to a value in the range of 0.1–0.5 $nS \cdot cm^{-2}$ (Table I).

The albumin was fatty-acid free ( < 0.005%) (Sigma No. A 0281) and was applied to both sides of the membranes at concentrations ranging from 0.1 to 0.2 $mg \cdot ml^{-1}$ (about 2 $\mu$M). Higher concentrations of albumin usually caused the membranes to rupture. The albumin concentration was sufficiently low that it had no significant effect on the pH or buffer capacities of the aqueous solutions. In one experiment we included EDTA (50 $\mu$M) to test for heavy-metal contamination of the protein, but no differences were observed.

A possible explanation for the albumin effect is that albumin removes a membrane component which causes $G_{H/OH}$. If so, then it might be possi-

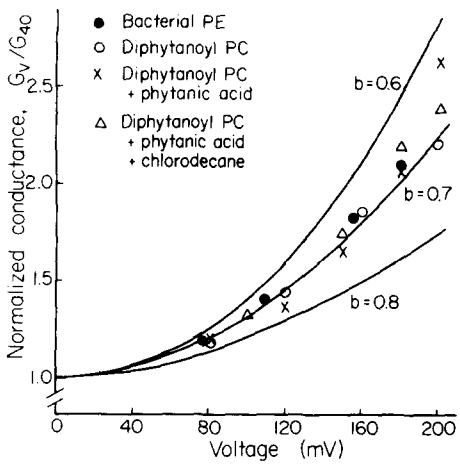


Fig. 5. Voltage dependence of the normalized steady-state conductance of four types of membranes. Solid lines are calculated from Eqn. 1, which describes charge transport through a trapezoidal energy barrier, and $b$ is the fraction of the membrane spanned by the minor base of the trapezoid. The phytanic acid mole fraction was 0.20, and the chlorodecane concentrations was 30% (v/v). The pH range was 6–12. Data obtained at lower pH ( < 4) gave a poorer fit, i.e., less voltage dependence. Each point is the average of at least three measurements.

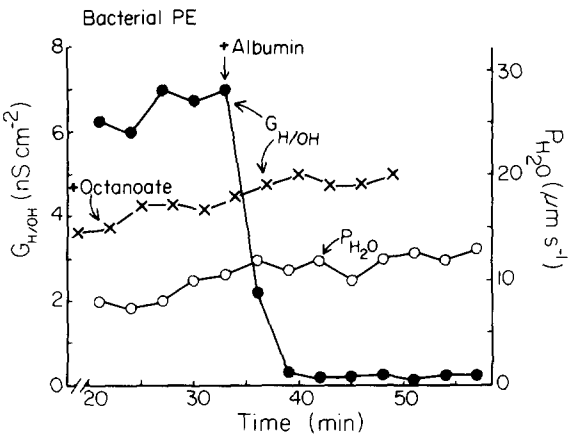


Fig. 6. Effects of serum albumin and octanoate on $H^+/OH^-$ conductance and water permeability of a bacterial PE decane membrane. The data shown by circles ($G_{H/OH}$ and $P_{H_2O}$) are from a single membrane. In a separate experiment (×), sodium octanoate (2 mM) was included in the aqueous solutions. In both experiments, serum albumin (fatty-acid free, 0.2 mg/ml) was added approx. 33 min after membrane formation. The exact time-course of albumin inhibition is uncertain, because several minutes are required to inject albumin (rear compartment) and perfuse with albumin containing solutions (front compartment). The pH values were 8.1 (rear) and 7.4 (front), buffered with Hepes plus Tris ($I = 0.025$, osmolarity = 0.08).

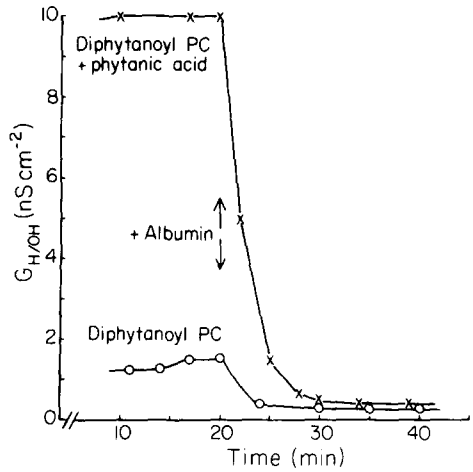


Fig. 7. Effects of serum albumin on $H^+/OH^-$ conductance through membranes made of diphytanoylPC or diphytanoylPC plus phytanic acid (10% w/w). Albumin was added approx. 20 min after membrane formation. Other conditions were as described for Fig. 6.

ble to block the albumin inhibition of $G_{H/OH}$ by saturating the binding sites with a competing ligand. For example, octanoate (2 mM) had no effect on $G_{H/OH}$ but blocked the ability of albumin to inhibit $G_{H/OH}$ in bacterial PE membranes (Fig. 6). Qualitatively similar results were obtained with diphytanoylPC and with phytanic acid containing membranes (data not shown).

Phloretin also inhibited $G_{H/OH}$ in unmodified membranes, phytanic-acid containing membranes and chlorodecane containing membranes (Table I and Fig. 8). The primary effect of phloretin is to decrease the membrane dipole potential, thus inhibiting anion conductance and enhancing cation conductance [38,39]. Thus, the inhibition of $G_{H/OH}$ by phloretin suggests that the $H^+/OH^-$ charge carrier is primarily anionic. As a control, we also tested the effects of phloretin (0.1 mM) on the conductance of a lipophilic cation, tetraphenylarsonium (1.0 mM), and found a $10^3$-fold increase (17 to 17200 nS · cm$^{-2}$), similar to the results of others [38].

Glycerol also inhibited $G_{H/OH}$ in both unmodified and phytanic acid containing membranes (Table I and Ref. 11). At a glycerol concentration of 9.6 M (75% w/w), the inhibition was about

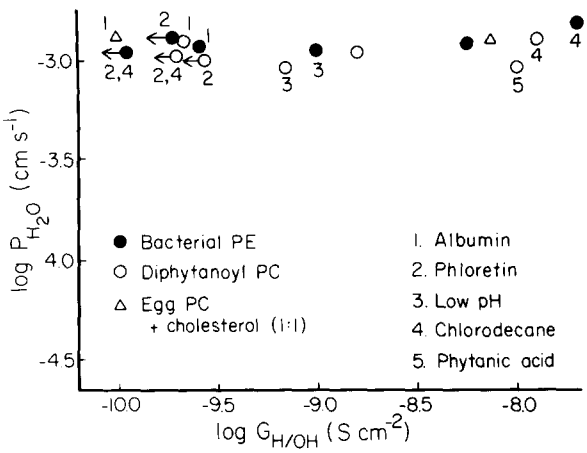


Fig. 8. Relation between water permeability and $H^+/OH^-$ conductance in several types of membranes under several conditions. The lipid compositions are indicated by symbols, and the modifying agents are indicated by numbers. The low pH experiments were done in HCl (pH 1.5–1.8). The other agents are described in Table I. Leftward arrows indicate that the value of $G_{H/OH}$ is an upper limit.

90%. In one experiment we added EDTA (50 $\mu$M) to check for possible heavy metal contamination of the glycerol, but no differences were observed. Additional data on glycerol inhibition of $G_{H/OH}$ are given in Ref. 11.

*Relation between water permeability and $G_{H/OH}$*

One possible mechanism for $H^+/OH^-$ transport through phospholipid bilayers involves proton jumping along hydrogen-bonded chains of water molecules which extend into the hydrophobic region of the membrane [1,2]. Thus, we wished to see if water permeability and $H^+/OH^-$ conductance were related. Fig. 8 shows that $P_{water}$ and $G_{H/OH}$ were not correlated under the conditions of our experiments. We did not attempt to measure water permeabilities in the presence of glycerol due to the higher viscosity and larger unstirred layer corrections required in glycerol-containing solutions.

## Discussion

*Mechanism of $H^+/OH^-$ conductance induced by phytanic acid*

Addition of phytanic acid to lipid bilayer membranes causes a $G_{H/OH}$ which increases with pH, phytanic acid concentration, membrane voltage and chlorodecane (Figs. 3–5, Table I). The $G_{H/OH}$ induced by phytanic acid is inhibited by phloretin and serum albumin (Fig. 7, Table I).

The effect of pH on $G_{H/OH}$ (Fig. 3) suggests that phytanate ($A^-$) plays a key role in $H^+/OH^-$ conductance. The effects of chlorodecane (dielectric constant) (Fig. 4) and membrane voltage (Fig. 5) are consistent with this notion. Furthermore, the first power dependence of $G_{H/OH}$ on phytanic acid concentration at constant pH (Fig. 4) suggests that phytanic acid may be acting as a simple ($A^-$ type) proton carrier. This could also explain the inhibition of $G_{H/OH}$ by phloretin (Table I).

A well known example of an $A^-$ type proton carrier is CCCP, a weak acid uncoupler of oxidative phosphorylation [27,43]. Like phytanic acid, CCCP causes a $G_{H/OH}$ which is proportional to the first power of the weak acid concentration at constant pH [43]. Furthermore, $G_{H/OH}$ increases with pH and shows a broad peak on the alkaline side of the p$K$, due to the much higher permeability of HA compared to $A^-$ [27,43]. In phytanic acid containing membranes, the pH dependence above the p$K$ is poorly defined, due primarily to the instability of the membranes at high pH *. Hopefully, future studies with other fatty acids or different lipid mixtures will provide a clearer test of the $A^-$ model at high pH **. However, the present data are sufficient to exclude the more

* Fatty acids in dilute aqueous solutions have p$K$'s near 4.8. However, fatty acids in bilayers have apparent p$K$'s which are much higher. For example, stearic acid (1.2 mol %) in PC vesicles has an apparent p$K$ about 7.0 [40], and oleic acid (5 mol %) in PC vesicles has an apparent p$K$ about 7.5 [41]. The increase in fatty acid p$K$ is due in part to the lower dielectric constant in the membrane surface (see Ref. 42 for discussion and references). Also, ionization of fatty acids in the membrane produces a negative surface charge which broadens the titration curve and contributes to the increase in apparent p$K$ [40].

** At pH $\gg$ p$K$, the predicted behavior of phytanic acid differs from CCCP. At high pH, the CCCP anion ($A^-$) becomes the predominant current carrying species, because $A^-$ has a relatively high permeability and is present in the aqueous solutions [27,43]. In contrast, phytanic acid is added to the lipid solution and is presumably confined to the membrane. Thus the steady-state conductance cannot be due to phytanate transport, even when pH $\gg$ p$K$.

complicated $HA_2^-$ carrier model, which shows a quadratic dependence of $G_{H/OH}$ on weak acid concentration [27,44,45].

The ability of serum albumin to inhibit phytanic acid induced $G_{H/OH}$ (Fig. 7, Table I) probably reflects the removal of phytanic acid from the membrane. Albumin binds a variety of fatty acids [34–37], including phytanic acid [46]. A reversible, pH-dependent transfer of fatty acids between albumin and phospholipid vesicles has been described by Hamilton et al. [47]. Their results, as well as ours, raise some interesting questions for future study. For example, how does albumin remove the fatty acids from the membrane? Does albumin facilitate transport of fatty acids across membranes?

In conclusion, our data are consistent with the hypothesis that phytanic acid acts as a simple proton carrier in lipid bilayer membranes. However, compared to classical proton carriers, phytanic acid is an extremely weak protonophore. In order to produce a proton conductance of 10 $nS \cdot cm^{-2}$ in phospholipid-decane membranes, the phytanic acid mol fraction must be $> 10^{-1}$ (Fig. 4), compared to $< 10^{-5}$ for FCCP (calculations based on data in Ref. 32). The high potency of classical protonophores such as CCCP and FCCP is due in part to the presence of $\pi$ electrons, which delocalize the charge and increase the $A^-$ (or $HA_2^-$) solubility in the low dielectric membrane interior [27]. Thus, phytanic acid is neither expected nor observed to be a highly effective proton carrier.

Nevertheless, under unusual conditions, such as in Refsum's (phytanic acid storage) disease, the levels of phytanic acid in blood and tissues may be very high, e.g., 0.6–3 mM in plasma and over 50% of the total fatty acids in kidney and liver lipids [16,17,48]. Furthermore, certain lesions such as neuropathy and cardiac arrhythmia appear to be closely linked to the plasma phytanic acid concentration [17]. In light of the fact that the $H^+/OH^-$ conductance of phytanic acid containing bilayers (Fig. 4) is of the same order of magnitude as some biological membranes, e.g., mitochondria [27,31], it seems reasonable to suggest that phytanic acid may alter proton electrochemical gradients across cell or organelle membranes. This might explain, for example, why cultured neurons exposed to phytanic acid have swollen mitochondria [49]. However, others have suggested that the primary membrane lesion in Refsum's disease may be an altered sterol composition rather than a fatty acid defect [50].

*Mechanism(s) of $H^+/OH^-$ conductance in unmodified phospholipid bilayers*

Our results confirm and extend the previous studies of Nichols and Deamer [1,2], and others [3–13] who found surprisingly high $H^+/OH^-$ permeabilities and/or conductances in phospholipid bilayers. The pH dependence of $G_{H/OH}$ is also consistent with previous observations on egg PC vesicles [2,8], although the range of pH shown in Fig. 3 is larger than that used in previous studies. In all studies, however, the change in $H^+/OH^-$ conductance (or net flux) is much smaller than the change in $H^+/OH^-$ concentrations. Thus, $H^+$ and/or $OH^-$ are not crossing the membrane by simple diffusion [2,8].

Since the transport mechanism(s) is unknown, and since the calculated $H^+/OH^-$ permeability coefficients are extremely pH dependent [2,8,11], different membrane systems can be compared only at the same pH, preferably at pH 7 [8,15]. In order to compare our $H^+/OH^-$ conductances with the $H^+/OH^-$ 'net' permeability coefficients calculated by others, we used the relation, $P_{H/OH} = RTG_{H/OH}/F^2c_{H/OH}$ where $P_{H/OH}$ is the net permeability and $c_{H/OH}$ is the $H^+$ or $OH^-$ concentration at pH 7. Thus, we obtain values of about $1 \cdot 10^{-5}$ $cm \cdot s^{-1}$ for bacterial PE and $0.4 \cdot 10^{-5}$ $cm \cdot s^{-1}$ for diphytanoylPC. Our values are lower than most, but still within the range reported for various types of phospholipid vesicles at neutral pH [1–15].

Ionic conductance and permeability mechanisms in unmodified lipid bilayers are poorly understood and difficult to study. Both the $H^+/OH^-$ and alkali/halide ion transport mechanisms remain to be identified. At neutral pH, our calculated $P_{H/OH}$ is at least five orders of magnitude higher than the alkali or halide ion permeabilities [1,15,51]. However, due to the extreme pH dependence of $P_{H/OH}$, this discrepancy disappears at pH 1–2, where the calculated $P_H$ is in the range of $10^{-12}$–$10^{-10}$ $cm \cdot s^{-1}$ [11], similar to $P_{Na}$ and $P_{Cl}$ in the planar bilayer system. At low pH in the

presence of $Cl^-$, large fluxes of HCl occur [51]. However, HCl diffusion cannot explain the 'electrogenic' $H^+/OH^-$ fluxes at neutral pH because, first, the HCl flux is nonconductive (electroneutral) [51], and, second, the $H^+/OH^-$ flux is either not $Cl^-$ dependent [2] (or weakly $Cl^-$ dependent [13]).

By comparing the properties of unmodified and fatty acid containing membranes, we hoped to gain insights into possible $H^+/OH^-$ transport mechanism(s). The similarities between the behavior of $G_{H/OH}$ in unmodified and fatty acid containing membranes (Figs. 2, 3, 5–7 and Table I) suggests that our lipids may contain weakly acidic contaminants which cause part of $G_{H/OH}$. The pH dependence, voltage dependence, and the response of $G_{H/OH}$ to chlorodecane, albumin, albumin plus octanoate, phloretin and glycerol are consistent with the weak acid hypothesis. Furthermore, the positive intercepts in Fig. 4 could be explained by the presence of about 1% (w/w) (about 2 mol%) phytanic acid in the diphytanoylPC.

Lipid oxidation and/or hydrolysis produces a variety of weakly acidic products [24,52–55], and even 'pure' phospholipids often contain fatty acids or other degradation products at levels of 1% or less [56]. Cafiso and Hubbell [8], who obtained very low values of $P_{H/OH}$, showed that 2% oxidation of the double bonds in egg PC causes a 15-fold increase in $P_{H/OH}$. Rossignol et al. [5], who obtained rather high values of $P_{H/OH}$, measured a 0.4% oxidation level in their phospholipid mixtures. Several investigators have noticed small amounts of negatively charged components in phosphatidylethanolamine at neutral pH [24,57] (discussed in Ref. 58). However, the 2–5-fold higher $G_{H/OH}$ of bacterial PE bilayers (Fig. 3) does not necessarily imply a higher level of contaminants, because the dipole potential is more positive in PE than in PC bilayers [30].

However, if lipid bilayers do contain weakly acidic 'endogenous protonophores', then their contribution to $G_{H/OH}$ is apparently superimposed upon a 'baseline' or 'background' $G_{H/OH}$ in the range of 0.1–1.0 $nS \cdot cm^{-2}$. In Fig. 3, for example, there is little difference between controls and phytanic acid containing membranes below pH 4. Thus, $G_{H/OH}$ at low pH appears to be unrelated to weak acid contaminants. Furthermore, in both controls and fatty-acid containing membranes, serum albumin reduces $G_{H/OH}$ to a baseline value of 0.1 to 0.5 $nS \cdot cm^{-2}$, regardless of the initial level of $G_{H/OH}$ (Table I, Figs. 6–8 and unpublished data).

This background $G_{H/OH}$ is very difficult to study in phospholipid-decane membranes. Perhaps future studies could utilize chlorodecane as an 'amplifier', assuming that no new conductance pathways are introduced by chlorodecane. Alternatively, very small $H^+/OH^-$ currents can be studied in phospholipid vesicles, for which a highly sensitive spin-labeled phosphonium probe has been developed [8,12]. With this technique, $H^+/OH^-$ currents in small (sonicated) egg PC vesicles were found to be enhanced by chloroform and halothane but, surprisingly, not affected by phloretin [8,12]. However, $P_{H/OH}$ in these small vesicles is about $2 \cdot 10^{-6}$ $cm \cdot s^{-1}$ [12] ($G_{H/OH} = 0.7$ $nS \cdot cm^{-2}$), within the range of our baseline values (Fig. 3 and Table I). Thus, the phloretin insensitivity might reflect the properties of a second, low conductance mechanism for $H^+/OH^-$. Further work is necessary to test this possibility.

*$H^+/OH^-$ conductance via 'water wires' or hydrated defects*

Another proposed mechanism to explain $G_{H/OH}$ is that $H^+/OH^-$ transport occurs along hydrogen-bonded chains of water molecules (water wires) which extend into the hydrophobic region of the membrane [1,2,7]. This mechanism is similar to the Grotthus conduction in water and ice [59,60]. The water wire, like the weak acid, provides a conductance mechanism for $H^+/OH^-$ that is not available to other inorganic ions.

Two possible routes exist for water wires, i.e., through the hydrophobic region or through transient 'hydrated defects' in the membrane structure. Hydrated defects have been proposed to explain the high $H^+/OH^-$ permeability of bilayers [15], as well as the selectivity among small, polar nonelectrolytes [61] and the permeability to alkali and halide ions [11,62] (but cf. Ref. 63).

With regard to $H^+/OH^-$ transport, our results show that $G_{H/OH}$ is sensitive to both the dipole potential (phloretin) and the dielectric constant (chlorodecane) (Table I, Fig. 8). Thus, if $H^+/OH^-$ transport occurs via water wires, their location is

apparently in the nonpolar region of the membrane, rather than in transient hydrated defects. We have shown elsewhere that selectivity among small, polar nonelectrolytes also resides in the nonpolar region [64]. Alkali/halide ion selectivity, on the other hand, appears to be more sensitive to the surface charge than to the dipole potential [65–67]. Thus, a hydrated defect pathway remains a possibility for alkali/halide ion conductance (as well as for the 'background' $H^+/OH^-$ conductance).

Although the water wire model is conceptually attractive, the results of this study raise several questions that are difficult to answer by means of the water wire model. First, why does $G_{H/OH}$ increase as $[H^+]$ decreases (Fig. 3), despite the fact that the mobility of $H^+$ is greater than $OH^-$ in both water and ice [60]? Second, why does serum albumin inhibit $G_{H/OH}$, and why does octanoate block the albumin effect (Figs. 6 and 8, Table I)? Third, why does phloretin inhibit $G_{H/OH}$ (Fig. 8, Table I)? Fourth, why do the conditions which enhance or inhibit $G_{H/OH}$ have no effect on water permeability (Fig. 8)? If water is involved in $H^+/OH^-$ conductance, then it apparently is not the same water which is involved in neutral water transport.

Previous evidence for the water wire model was based mainly on temperature studies which showed, first, rather high activation energies for $P_{H/OH}$ ranging from 13 to 20 kcal · $mol^{-1}$ [4–6,13], second, large decreases in both $H^+/OH^-$ and water permeabilities when phospholipids undergo a liquid-crystal to gel phase transition [6] and, third, the absence of a permeability maximum for both $H^+/OH^-$ and water at the phase transition temperature [6]. However, these findings are also compatible with the weak acid model, because, first, transmembrane diffusion rates of many nonelectrolytes and hydrophobic ions show activation energies in this range [68–73], second, phase changes usually cause dramatic changes in their permeabilities or conductances [71–73] and, third, hydrophobic ions apparently do not show a permeability or conductance maximum at the phase transition temperature [72–74].

Previously we thought that the voltage sensitivity of $G_{H/OH}$ (Fig. 5) was inconsistent with the water wire model [11]. However, Nagle [75] has shown that $H^+/OH^-$ transport via water wires may give superlinear current-voltage curves under some conditions, depending upon the rate limiting step. Thus, the data in Fig. 5 may be consistent with both the water wire and weak acid models.

The glycerol inhibition of $G_{H/OH}$ may also be consistent with both the water wire and weak acid models. In addition to reducing water activity in the bulk solutions [11] and substituting for water in the polar headgroups [76], glycerol also reduces lipid fluidity in both central and peripheral regions of the bilayer [77]. Glycerol also reduces the membrane dipole potential (Ref. 78 and Simon, S.A., personal communication). Thus, glycerol inhibits various types of carrier-mediated transport, including $H^+$ transport by 2,4-dinitrophenol [79] and phytanic acid (Table I). The fact that 9.6 M glycerol causes the same percent inhibition (approx. 90%) in both unmodified and phytanic acid containing membranes (Table I) is consistent with the notion of similar conductance mechanisms in both types of bilayers. However, further work is necessary to determine exactly how glycerol inhibits $G_{H/OH}$.

## Conclusions

In conclusion, we have examined two models for $H^+/OH^-$ conductance and permeability, i.e., the weak acid (endogenous protonophore) model and the hydrogen-bonded water strand (water wire) model. For phytanic acid containing bilayers, a weak-acid carrier model can explain most of the observed $G_{H/OH}$. For unmodified membranes, neither model can be decisively rejected, because the weak acids are unidentified and the properties of the water wires are unknown. However, at pH > 4, most of $G_{H/OH}$ appears to be due to weakly acidic contaminants in the phospholipids. At low pH, or in the presence of albumin or glycerol, a significant $G_{H/OH}$ remains. This pH-independent $G_{H/OH}$ can be explained by a water-wire model [75].

Whether endogenous protonophores or water wires are important in biological membrane permeability remains to be seen. However, in some biological membranes, $P_H$ is orders of magnitude higher than $P_{Na}$ [80]. For example, in renal brush border membranes the proton permeability is 5 ·

$10^{-3}$ cm · $s^{-1}$, and proton diffusion utilizes a lipid pathway which is independent of the pathway for water [81]. Furthermore, exposure of gastric microsomes to bovine serum albumin reduces $H^+$ permeability 2–3-fold without altering the rate of active $H^+$ transport (Hanzel, D.K. and Forte, J.G., personal communication). Finally 'washing' photoreceptor membrane vesicles with bovine serum albumin reduces $H^+/OH^-$ conductance by about two orders of magnitude (Ojcius, D.M., personal communication).

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