ELECTROGENIC H^+/OH^- MOVEMENT ACROSS PHOSPHOLIPID VESICLES MEASURED BY SPIN-LABELED HYDROPHOBIC IONS

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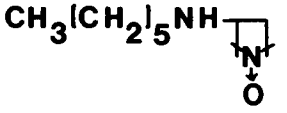
ABSTRACT Transmembrane pH gradients created across phospholipid vesicles give rise to time-dependent potentials as determined from the EPR spectra of phosphonium ion spin labels in the system. From the time-dependent spectra, the transmembrane H^+/OH^- current is obtained and hence the current-voltage curve for the vesicle membrane is obtained. The current-voltage curve is linear with a membrane resistance of $3 \pm 2 \times 10^9$ Ωcm^2 corresponding to a membrane permeability of $5 \pm 2 \times 10^{-7}$ cm/s. This unusually high permeability is further increased by small amounts of lipid oxidation, $CHCl_3$ or the general anesthetic halothane.

INTRODUCTION

The permeability of phospholipid bilayers to small inorganic ions is expected to be extremely low, due to the high energy required to remove the ion from water and place it into the low-dielectric membrane interior. This expectation is clearly met for common inorganic ions such as Na^+ and K^+, whose permeabilities are on the order of 10^{-12} to 10^{-14} cm/s (Hauser et al., 1972; Johnson and Bangham, 1969; Pike et al., 1982; Mimms et al., 1981). Recently, however, phospholipid bilayer permeabilities for protons (and/or hydroxide ions) have been reported which range from 10^{-3} to 10^{-9} cm/s (Nichols et al., 1980; Nichols and Deamer, 1980; Biegel and Gould, 1981; Clement and Gould, 1981; Nozaki and Tanford, 1981; Gutknecht and Walter, 1981; Rossignol et al., 1982; Deamer and Nichols, 1983; Elamrani and Blume, 1983). Neither the mechanism of the conductance nor the reason for the wide variation in reported values has been satisfactorily elucidated at this point.

In the present paper, we report a new approach to the estimation of transmembrane ionic currents in phospholipid vesicles and apply it to the study of electrogenic proton transport in sonicated vesicles formed from egg phosphatidylcholine and diphytanoylphosphatidylcholine. The method for studying ion conductance used here makes use of the membrane-permeable phosphonium spin label shown below, that we refer to as (I). In the presence of



(I)

phospholipid vesicles, the EPR spectrum of (I) reveals the presence of both membrane bound and free (aqueous) populations, and the amplitude of the high field resonance line, A, may be used to determine the binding constant of (I) to the membrane (Cafiso and Hubbell, 1981). In previous work, we have shown that the apparent binding constant of (I) is a function of the equilibrium transmembrane potential, and the relationship between these quantities is accurately described by a Nernst equation modified to include the weak binding of (I) to the membrane (Cafiso and Hubbell, 1981).

The rate of relaxation of transmembrane gradients of (I) has also been investigated in vesicles as a function of transmembrane potential, and the rate is adequately described by a simple Eyring model with a single potential-dependent barrier height (Cafiso and Hubbell, 1982). In the present work, we extend the application of the phosphonium (I) to the measurement of time-dependent potentials generated by the flux of H^+ and/or OH^- ions across the membrane, and show how this information can be used to obtain the net membrane current and hence the current-voltage characteristic for the phospholipid vesicle.

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EXPERIMENTAL

Materials and Methods

The spin-labeled phosphonium (I) and *N*-tempoyl-*N*-hexylamine (II) were synthesized as previously described (Cafiso and Hubbell, 1978 *a,b*). Egg phosphatidylcholine (PC) was prepared according to the procedure of Singleton et al. (1965) and stored in chloroform at −20°C under an argon atmosphere. Diphytanoyl PC was obtained from Avanti Biochemicals (Birmingham, AL). To prepare phospholipid vesicles, aliquots of PC in chloroform were dried under a stream of nitrogen and vacuum dessicated for a minimum of 15 h at $5 - 10 \times 10^{-5}$ torr at room temperature. The lipids were dispersed in the appropriate buffer solutions and sonicated as previously described (Cafiso and Hubbell, 1978*a*). Vesicles formed from egg PC had an average diameter of ~300 Å, as determined by platinum shadowing and negative-stain electron microscopy (Castle and Hubbell, 1976). Vesicles formed from diphytanoyl PC had an average diameter of ~550 Å.

Gradients of pH were established across these membrane vesicles by either diluting the vesicle suspension into the appropriate buffer or by adding base to the existing vesicle-buffer suspension. In some cases, it was necessary to create these pH gradients rapidly in the EPR cavity. To accomplish this, the rapid mixing device described earlier was used (Cafiso and Hubbell, 1982). All experiments were performed at room temperature.

The lipid concentration of the vesicle suspension was determined by phosphate assay using a modification of the procedure of Bartlett (1959). The lipid concentration and the vesicle sizes were used to determine V_o/V_i, the ratio of external/internal volumes, and V_{mo}/V_{mi}, the ratio of external/internal volumes of the membrane surface binding domains (Cafiso and Hubbell, 1978*a*).

The free-signal amplitudes of spin labels I and II were used to determine the partitioning of these labels as described previously (Cafiso and Hubbell, 1981). Both transmembrane potentials (from I) and pH gradients (from II) were computed from this spectral parameter as described previously (Cafiso and Hubbell, 1978*a,b;* 1981).

RESULTS

Electrogenic Transmembrane H^+/OH^- Movement Can Be Detected using Paramagnetic Hydrophobic Ions

When a pH gradient is established across phospholipid vesicles in the presence of (I), the high-field resonance amplitude, *A*, becomes time dependent, indicating a time-dependent redistribution of (I) in the system (Cafiso and Hubbell, 1981; 1982). Fig. 1 *a* shows a tracing of *A* vs. time when a ΔpH of 1.04, inside acidic, is created across the vesicle membranes at time zero. The decreasing amplitude implies an increased binding of (I) with time, and, as shown earlier, this indicates the development of an inside-negative transmembrane potential (Cafiso and Hubbell, 1978*a*). The only condition necessary for this conclusion to hold is that no significant chemical reduction of the nitroxide function takes place, and in all experiments reported here this is the case. The membrane potential can only arise in this system as a result of the net transmembrane flow of an ionic species down a chemical potential gradient.

After generation of the transmembrane pH gradient, there were spatial gradients of several ions across the membrane, namely H^+, OH^-, Na^+, K^+, and the various ionic and zwitterionic species of the buffers used. Na^+ and K^+ are simply too impermeable to give rise to transmembrane diffusion potentials on the time scale shown here (Hauser et al., 1972; Johnson and Bangham, 1969; Pike et al., 1982), and it is a relatively straightforward matter to show that this also applies to the ions and zwitterions of the buffers used (morpholinopropanesulfonic acid [MOPS], morpholinoethanesulfonic acid [MES], and phosphate). For example, vesicles with any one of the buffers in the vesicle interior and any of the other buffers in the external medium at the same pH and concentration did not develop detectable diffusion potentials. Thus we conclude that the resultant potentials are due to an electrically active H^+/OH^- flux. In further support of this conclusion, the equilibrium transmembrane potentials, $\Delta\psi$, calculated from the final spectral amplitudes were always in close agreement with the predicted Nernst potential for the existing H^+/OH^- gradient. For example, for the data in Fig. 1, the predicted equilibrium potential was 62 mV, and we experimentally observed 65 ± 3 mV. The equilibrium diffusion potentials due to H^+/OH^- gradients were extremely stable in time, decaying <7% in a 24 h period, suggesting the relative impermeability of all other ions concerned. From the amplitude-time data in Fig. 1 *a*, the corresponding potential-time curve can be computed (Cafiso and Hubbell, 1978*a*) and is shown in Fig. 1 *b*. Calculation of the membrane potential from the spectral amplitude data was based on the assumption that the phospho-

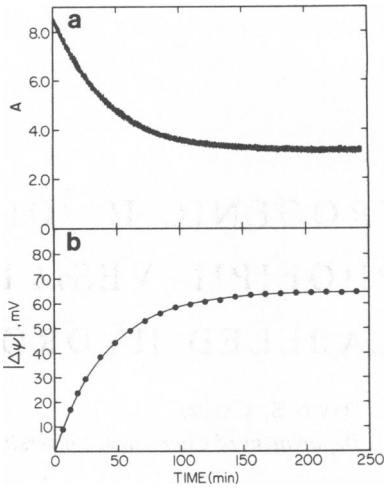


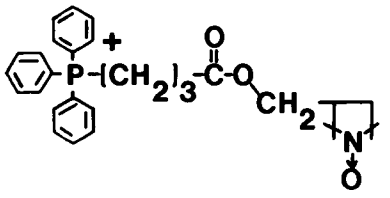
FIGURE 1 (*a*) A tracing of the high-field resonance amplitude, *A*, of 20 μM phosphonium (I) in the presence of sonicated egg PC vesicles as a function of time following the establishment of a transmembrane pH gradient. The pH gradient was created by mixing 52 μl of vesicle suspension prepared in 100 mM MES, pH 6.51, with 40 μl of 100 mM MOPS, pH 8.50. The final external pH was 7.55. In addition, these buffers contained 125 mM Na_2SO_4. Egg PC was at a final concentration of 52 mg/mL with 1 μM ϕ_4B^-. A_f^o, the total free-signal intensity for 20 μM (I) in the absence of vesicles is ≈21.5 on this scale. (*b*) Points (●) are values of $|\Delta\psi|$ calculated from the data in *a* (see text). The solid line (—) is an exponential fit, $|\Delta\psi(t)| = 65(1 - e^{-.024t})$.

nium transmembrane movement is not rate limiting; that is, the phosphonium must at all times be in equilibrium with the developing transmembrane potential.

Because the time constant for the relaxation of a transmembrane gradient of (I) is approximately an order of magnitude shorter than that shown for the decay of A in Fig. 1 (Cafiso and Hubbell, 1982), the above assumption is expected to be approximately correct. To test this, we have taken advantage of the fact that small amounts of tetraphenylboron (ϕ_4B^-) give rise to a dramatic decrease in the transmembrane relaxation time constant for (I) (Cafiso and Hubbell, 1982). Fig. 2 shows the effects of ϕ_4B^- on the initial rate of amplitude change of (I) in the presence of vesicles across which a H^+/OH^- gradient was established. As shown, concentrations of ϕ_4B^- as low as 0.5 μM (<1 ϕ_4B^- per 20 vesicles) cause a measurable increase in the rate of decay of A, but from 0.5–5 μM ϕ_4B^-, the time course of A is completely independent of the concentration. Over this same range, the rate constant for the transmembrane movement of (I) increases by an order of magnitude (Cafiso and Hubbell, 1982). Thus, in the presence of small amounts of ϕ_4B^-, it appears that the phosphonium movement certainly cannot be rate limiting, and Fig. 1 *b* is expected to be an accurate representation of the voltage-time curve. All data in this paper were obtained in the presence of 1 μM ϕ_4B^-. These results also demonstrate that ϕ_4B^- is itself not a proton ionophore.

It is important to note here that, due to the small concentration of phosphonium relative to phospholipid (1:5,000), the net charge transport due to the phosphonium itself is quite small and will not significantly perturb the potential determined by the H^+/OH^- flow (Cafiso and Hubbell, 1982). This expectation is experimentally confirmed by the fact that the time course of approach to equilibrium as well as the equilibrium value of A is independent of the concentration of (I) in the range of 2×10^{-6} to 2×10^{-4} M (data not shown). This also demonstrates that (I) itself is not a carrier of OH^- ion.

If the pH gradient across the vesicle membrane remains constant during the approach to equilibrium, the voltage-time curve shown in Fig. 1 *b* corresponds to the simple charging of a series resistance-capacitance circuit with a battery of voltage equal to the Nernst equilibrium potential for protons. To check the constancy of the H^+/OH^- gradient during the build-up of $\Delta\psi$, the secondary amine spin label, (II) below, was used.



(II)

The partitioning of this spin label has been shown to be a sensitive function of transmembrane H^+/OH^- gradients in suspensions of phospholipid vesicles (Cafiso and Hubbell, 1978*b*), and the effective partition coefficient is related to the high field amplitude, A, of the spectra, just as for the phosphonium (I). A recording of A vs. time for (II) is shown in Fig. 3 after the establishment of a pH gradient at time zero. The conditions were identical to those in Fig. 1 except that buffer concentrations of both 100 and 10 mM were used. At 100 mM buffer, the conditions corresponding to the data in Fig. 1 *a*, the pH gradient remained essentially constant during the time course of the potential build-up. These pH gradients were also extremely stable in time, suggesting the absence of significant electroneutral

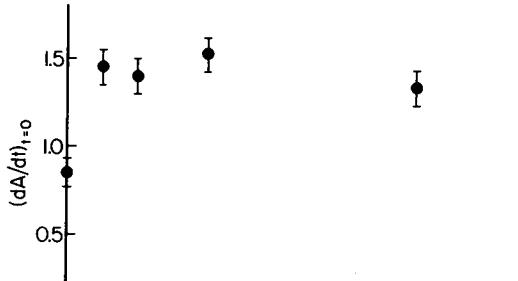


FIGURE 2 The initial rate of change of the high-field resonance amplitude for the phosphonium (I) following the establishment of a pH gradient plotted vs. total ϕ_4B^- concentration. In each case, the pH gradient was created by adding the appropriate volume of 1.0 M NaOH to a vesicle suspension prepared in 125 mM MES, pH = 5.5, to give a final external pH of 7.0. In most cases ϕ_4B^- had no effect on $(dA/dt)_{t=0}$ or the time course of A. In other cases, as shown here, 0.5 μM ϕ_4B produced a maximal effect. Egg PC was at a final concentration of ≈50 mg/ml with 20 μM phosphonium (I).

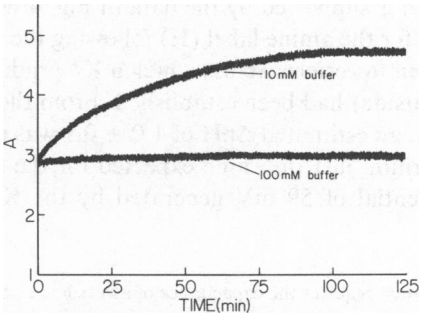


FIGURE 3 Tracings of the high-field resonance amplitude, A, of (II) in egg PC vesicles vs. time following the creation of a transmembrane pH gradient ($\Delta pH \simeq 1.1$). For the trace labeled 100 mM, the pH gradient was created by mixing 52 μl of vesicles prepared in 100 mM MES, pH 6.5, with 48 μl of 100 mM MOPS, pH = 8.5. The final external pH was 7.6. In addition, these buffers contained 125 mM Na_2SO_4. For the trace labeled 10 mM, the pH gradient was created in the same way, except that all buffer concentrations were 10 mM and each contained 12.5 mM Na_2SO_4. Sonicated egg PC was at a final concentration of 52 mg/ml. Spin label (II) was at a concentration of 20 μM and A_f^0, the total free signal intensity in the absence of vesicles, was 21 units on this scale. At 100 mM buffer, initial and final Δ identical. At 10 mM buffer, initial and final ΔpH's are 1.11 and 0.70, respectively.

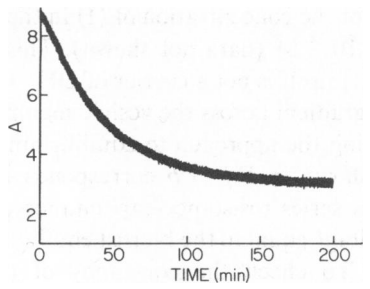


FIGURE 4 Tracing of the free signal amplitude, A, of (II) in egg PC vesicles with a transmembrane K^+ gradient following the addition of 5 μM valinomycin. Vesicles contained 250 mM K_2SO_4, 50 mM Na phosphate (pH 6.8) internal and 25 mM K_2SO_4, 225 mM Na_2SO_4 and 50 mM Na phosphate (pH 6.8) external, giving a ratio of K^+ internal/external of 10:1. At equilibrium ΔpH is 1.0 ± 0.1 as calculated from $A(\infty)$.

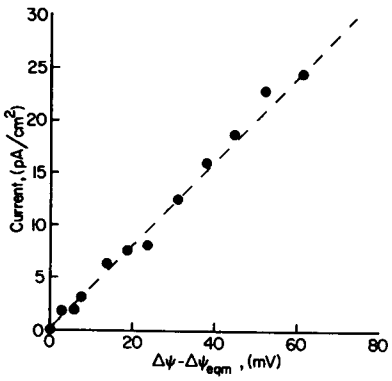


FIGURE 5 The current-voltage curve for sonicated egg PC vesicles constructed from the data in Fig. 1. The current was obtained from the slope of the voltage-time data in two ways. The dashed line is the current calculated from the derivative of the exponential fit to the voltage-time curve shown in Fig. 1 *b*. The points (●) represent currents obtained by direct numerical differentiation of the voltage-time data. Calculation of the current assumes a membrane capacitance of 0.9 μF/cm^2.

flow of H^+/OH^-. However, when the buffer capacity was lowered, the amplitude A and the H^+/OH^- gradients were then time dependent and decayed on the same time scale as $\Delta\psi$ was observed to appear. At 10 mM buffer, the H^+/OH^- movement that occurs to establish $\Delta\psi$ was sufficient to perturb the pH gradient. The ΔpH decrease deduced from the data in Fig. 3 and the known buffer capacity yielded an estimated 4.2 H^+ (OH^-) that have traversed each vesicle. If these move without the flow of compensatory charge, a $\Delta\psi$ of ~40 mV should result (assuming a membrane specific capacitance of ~0.9 μF/cm^2 [Montal and Mueller, 1972]).[1] The actual potential measured in this case by (I) is 43 ± 3 mV. The close agreement of these values strongly supports the idea of a purely electrogenic H^+/OH^- movement.

This being the case, pH gradients should develop upon the establishment of a transmembrane potential. This expectation is supported by the data in Fig. 4, which show A vs. time for the amine label (II) following the addition of valinomycin to vesicles across which a K^+ gradient of 10:1 (inside/outside) had been established. From the partitioning of (II), an estimated ΔpH of 1.0 ± 0.1 was established at equilibrium, just the value expected for the transmembrane potential of 59 mV generated by the K^+-selective conductance due to valinomycin.[2] These and the preceding results leave little doubt that electrogenic H^+/OH^- flows occur in this system.

The Membrane Current-Voltage Curve and Ionic Permeability Can Be Obtained from Time-dependent Phosphonium Partitioning

As shown above, partitioning changes in the phosphonium label (I) (following creation of ΔpH) provide a time-resolved estimate of potentials resulting from electrogenic proton (hydroxide) movement across sonicated egg PC vesicles. The first time derivative of this data, multiplied by the specific membrane capacitance, yields the net membrane current that in turn is used to construct the current-voltage curve shown in Fig. 5. The slope of this curve is constant and yields directly an integral membrane resistance of 3 ± 2 × 10^9 Ωcm^2. Notice the extremely small current density being measured here, on the order of a few picoamperes per square centimeter. This high sensitivity results from the large number of vesicles being observed (~10^{14}), and hence the large total number of spin-label molecules moving, even though the number per unit membrane area is very small. Current-voltage curves for the protonic flow are always linear in undoped vesicle membranes and remain linear for larger values of ΔpH as well. The membrane resistance is also surprisingly insensitive to the bulk concentration of H^+ (see below). The linearity of this curve is distinctive and is not consistent with a simple

[1]This calculation requires the capacitance of a vesicle; i.e., the product of the vesicle surface area and the membrane specific capacitance. The surface area chosen for this calculation is the geometric mean surface area, $4\pi r_o r_i$, where r_o and r_i are the outer and inner radii defining the hydrophobic boundary surfaces of the bilayer, respectively. This particular surface area is chosen for the following reason. The specific capacitance for a spherical capacitor is just $c = 4\pi r_o r_i \epsilon_0 \epsilon / (r_o - r_i)$, where ϵ_0 is the permittivity of free space and ϵ is the membrane dielectric constant. If the specific capacitance of the spherical shell is defined relative to the area $4\pi r_o r_i$, it is just $\tilde{c} = \epsilon_0 \epsilon / (r_o - r_i) = \epsilon_0 \epsilon / d$, where d is the membrane thickness. Note that this is the same as the specific capacitance of a planar capacitor of the same substance and thickness. Thus the same specific capacitance describes the spherical and planar capacitor if referred to the above described mean surface area for the sphere.

[2]With the vesicle concentration used here, the addition of 5 μM valinomycin in the absence of K^+ does not significantly alter the rate at which potentials develop following the establishment of a transmembrane pH gradient. Thus, in this system, valinomycin does not appear to contribute significantly to the movement of protons which occurs following the creation of a transmembrane potential.

thermally activated diffusion of a charged species across a single central membrane barrier.

As is usual, we define the phenomenological membrane permeability, P, as

$$P \equiv (J/\Delta C)_{\psi-0} = \frac{1}{ZF}(i/\Delta C)_{\psi-0} \quad (1)$$

where J is the membrane flux per unit area, i is the equivalent current density, ΔC is the transmembrane concentration difference of permeant species, Z is the valence of the permeant species and F is the Faraday constant. In this report, we do not distinguish between H^+ and OH^- flow, and define a net permeability as

$$P_{net} = P_{H^+} + P_{OH^-}. \quad (2)$$

This is also the quantity reported by Nichols and Deamer (1980) and will serve as a basis for comparison between the studies. The initial membrane current density, i_o (at zero potential), in this representation is

$$i_o = (\partial\psi/\partial t)_{\psi-0}\,\tilde{c} = F\{P_H([H^+]_i - [H^+]_o) - P_{OH}([OH^-]_i - [OH^-]_o)\} \quad (3)$$

where $\tilde{c}$ is the membrane specific capacitance. Under conditions where $[H_i^+]\,[H_o^+] = 10^{-14}$, this reduces to

$$i_o = (\partial\psi/\partial t)_{\psi-0}\,\tilde{c} = FP_{net}([H^+]_i - [H^+]_o). \quad (4)$$

The data in Fig. 1 closely correspond to these conditions ($pH_i = 6.51$; $pH_o = 7.55$), and using $\tilde{c} \simeq 0.9 \times 10^{-6}$ F/cm^2 one obtains from Eq. 4 and the initial slope of the potential-time curve a permeability of $P_{net} = 7 \times 10^{-7}$ cm/s. From 10 independent experiments, we find an average permeability of $P_{net} = 5 \pm 2 \times 10^{-7}$ cm/s.

An independent value for P_{NET} can be obtained from the initial time rate of change of internal pH in vesicles with a transmembrane pH gradient and a weak internal buffer. These data are available from Fig. 3 for vesicles with initial pH gradient of $\Delta pH = 1.1$ and 10 mM MES as an internal buffer. According to Eq. 1 and 4 above, P_{NET} for this situation can be expressed as

$$P_{NET} = \frac{B(\partial pH/\partial t)_{\Delta\psi-0} V_i}{A([H^+]_i - [H^+]_o)} = \frac{B(\partial pH/\partial t)_{\Delta\psi-0} r_i^2}{3r_o([H^+]_i - [H^+]_o)}$$

where B is the buffer capacity, V_i is the vesicle internal volume, and A is the vesicle geometric-mean surface area (see footnote 1), and r_i, r_o are the vesicle inner and outer radii, respectively. For 10 mM MES, B is found to be 5×10^{-3} M(pH)$^{-1}$ and taking $r_i = 100$ Å, $r_o = 150$ Å, we find $P_{NET} = 9.8 \times 10^{-7}$ cm/s, in reasonable agreement with that found from the time-dependent potential changes. Because we have done many more experiments of the latter type, our reported value for P_{NET} will be based on those measurements.

The linearity of the current-voltage (I-V) curve shown in Fig. 5 demonstrates that the H^+/OH^- flow is proportional to the transmembrane electric potential difference. To study the dependence of flow on the concentration gradient, the initial membrane current was studied as a function of the transmembrane concentration difference at constant ΔpH, i.e., at a constant H^+_{in}/H^+_{out}. The results are shown in Fig. 6. For the data shown here, the concentration difference of H^+ (and OH^-) varies by more than a factor of 10^5, while the initial flux (proportional to $\partial\psi/\partial t$) varies by only a factor of ~10. Clearly, the flux is not directly proportional to the concentration difference of either H^+ or OH^-.

Halogenated Hydrocarbons and Lipid Oxidation Enhance Electrogenic H^+/OH^- Movement

While the above data are representative of the results obtained, considerable variability in the protonic current was typical under experimental conditions that were not carefully controlled. Chloroform in trace amounts that remained with the lipids dramatically enhanced the protonic flow and was one factor that contributed to variability. Complete removal of chloroform from the phospholipids before vesicle formation was crucial to obtain reproducible, low, proton conductances. Vacuum dessication for a minimum of 15 h at 100 μmHg at 23°C was found to be necessary. Other halogenated compounds such as halothane produced similar effects. Due to interest in halothane as a clinical general anesthetic, we have studied it in more detail than chloroform, although the two are very similar quantitatively. Fig. 7 *a* shows the time rate of change of A and hence transmembrane potential after the creation of a $\Delta pH = 1.0$ gradient across vesicles that were at equilibrium with 2% halothane vapor (clinical anesthesia concentrations). Under these conditions, there is ~5

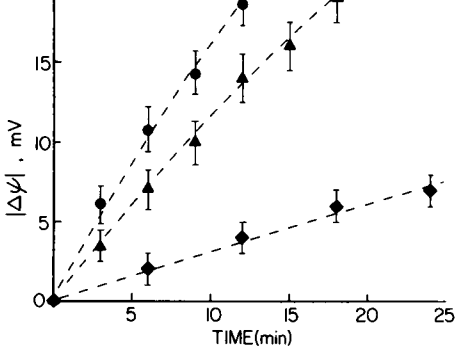


FIGURE 6 A plot of the voltage vs. time following the creation of different proton gradients across egg PC vesicles. In each case, $\Delta pH = 1.0$, the phosphonium (I) was at 20 μM, and ϕ_4B^- was added to 1 μM. $\Delta H^+ = [H^+_{in} - H^+_{out}]$. ●, $\Delta H^+ = 4.5 \times 10^{-9}$ M (pH = 8.3 inside, 9.3 outside); ▲, $\Delta H^+ = 8.0 \times 10^{-7}$ M (pH = 6.05 inside, 7.05 outside); ♦, $\Delta H^+ = 9.9 \times 10^{-4}$ (pH = 3.0 inside, 4.0 outside).

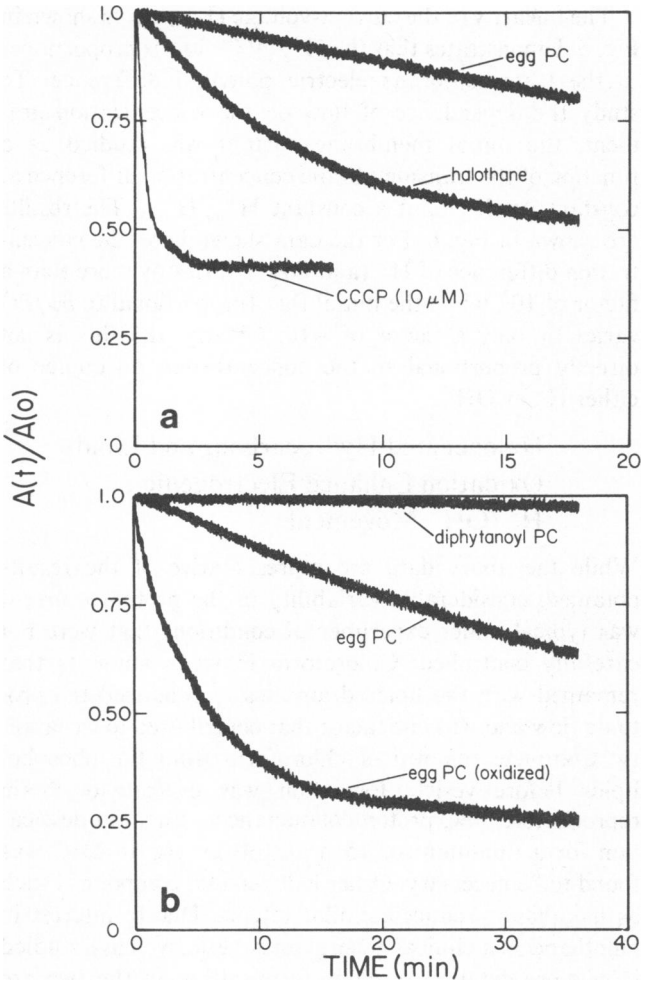


FIGURE 7 Normalized high-field resonance amplitude as a function of time for the phosphonium label (I) following establishment of a pH gradient ($\Delta pH = 1.0$) across egg PC vesicles. All vesicle concentrations were 50 mg/ml with 20 μM (I) and 1 μM ϕ_4B^-. In *a*, "pure" egg PC is compared with egg PC to which halothane (2% from vapor) or 10 μM CCCP is added. Equilibration with 2% halothane vapor produces approximately the same effect as 0.25% added $CHCl_3$ (by wt) and is close to concentrations that produce anesthesia. In *b* egg PC is compared with vesicles formed from nonoxidizable diphytanoyl PC and with egg PC, in which some of the unsaturated positions are oxidized. This oxidized egg PC has an "oxidation index" (A_{233}/A_{215}) of 0.47, which apparently corresponds to ~2% of the 1.6 double bonds per PC being oxidized (Klein, 1970). Note the different time scales in *a* and *b*.

mol % halothane in the bilayers. Also shown for comparison are the rates for vesicles without halothane and vesicles containing 10 μM of the proton ionophore carbonyl cyanide *m*-chlorophenylhydrazone (CCCP). Lipid oxidation is another factor that leads to variability in the H^+/OH^- permeability. As shown in Fig. 7 *b*, oxidation of a relatively small fraction of the double bonds in egg PC leads to a dramatic increase in the initial protonic flux. In this case, oxidation of <4% of the ~1.6 double bonds per PC molecule (as judged from a 233 nm absorption by the lipid [Klein, 1970]), leads to an ~15-fold increase in the initial flux. The synthetic lipid diphytanoyl PC forms fluid bilayers at room temperature (Lindsey et al., 1979) but has no potential oxidation sites. The H^+/OH^- flux across vesicles formed from the lipid is at least 30-fold less than that across egg PC vesicles under the same conditions (see Fig. 7 *b*). However, the flux of the phosphonium (I) itself in the presence of 1 μM TPB is only ~2–3 times slower, compared with egg PC (data not shown).

DISCUSSION

For slowly varying potentials across lipid vesicles (time constant greater than ~4 min) the time-dependent partitioning of the phosphonium spin label (I) may be used to estimate both the instantaneous membrane potential and the net membrane current. In the present case, the current is due to transport of H^+ and/or OH^-, but the method is quite generally applicable to the study of electrogenic fluxes of any ionic species. The time resolution of the technique could be greatly enhanced by the use of anionic hydrophobic spin labels such as nitroxide derivatives of tetraphenylboron, and work along this line is in progress. In any case, the time resolution of the phosphonium is clearly sufficient to allow investigation of the electrogenic H^+/OH^- flux. The purely exponential membrane charging curve (Fig. 1) indicates a voltage-independent membrane resistance to the charge-carrying species, since the driving force (ΔpH) remains constant and we assume that the capacitance is voltage independent. The constancy of the membrane resistance is graphically illustrated by the linearity of the membrane I-V curve (Fig. 2). A simple diffusion mechanism in which the rate-limiting step is thermally activated diffusion across an image energy barrier in the bilayer center gives rise to a highly nonlinear (exponential) I-V curve (Neumcke and Laüger, 1969; Hall et al., 1973; Andersen and Fuchs, 1975). This mechanism accounts well for the voltage dependence of the transport of hydrophobic ions, but clearly not for H^+/OH^- transport in "unmodified" bilayers as observed here. The constant membrane resistance of $3 \pm 2 \times 10^9$ Ωcm^2 is relatively high compared with that found for solvent-free unmodified planar bilayers, which fall in the range of 10^6–10^8 Ωcm^2 under similar conditions, although it is similar to the maximum value of the thicker solvent-containing bilayers (Haydon and Hladky, 1972). The wide variation of resistances found in the planar bilayers indicates the well-known sensitivity of this property to details of the preparation such as solvent content, impurities, and lipid oxidation level. These variables are relatively easy to control in vesicles and we have taken particular care to reduce the effect of these perturbants to a minimum, and the high resistance found is not unreasonable.

Another striking aspect of the H^+/OH^- transport is the lack of proportionality between charge flow and concentration gradient of either H^+ or OH^-. This behavior was also observed by Nichols and Deamer (1980) and interpreted as a change in membrane permeability with pH. This clearly must be the case if one adopts the phenomenologi-

cal definition of permeability defined in Eq. 1. This, however, does not imply a pH-dependent change in membrane structure or a change in mechanism with pH. The permeability as defined above is only a constant of the system for certain mechanisms, for example, simple diffusion. For more complex mechanisms, P may also become dependent on the concentration of transported species, as it is here. This supports the conclusion from the linear I-V curve that the transport mechanism is not simple thermally activated diffusion of H^+/OH^-. The form of dependence of charge transport on concentration gradient observed here could result from a mechanism in which protons were being translocated via a saturable carrier (Schultz, 1980).

A number of other laboratories have studied H^+/OH^- fluxes across phospholipid bilayers, and it is worthwhile to attempt a comparison between some of these studies and the present one. The experiments of Gould and co-workers (Clement and Gould, 1981; Biegel and Gould, 1981) are in principle the most directly comparable to ours, since these authors investigated H^+/OH^- fluxes across sonicated vesicles of asolectin and egg phosphatidylcholine. Although electric potentials were not directly measured, Clement and Gould cite indirect evidence that H^+/OH^- flow is electrogenic and reaches an electrochemical equilibrium across the vesicle membrane within ~1 s after the creation of a pH gradient. In the experiments reported in the present paper, however, this same process, as measured directly by the membrane potential, occurs with a half-time of ~40 min. The tremendous disparity in apparent H^+/OH^- transport rate is perhaps due to the presence of large amounts of $CHCl_3$ in the membrane studied by Clement and Gould (1981). The lipids used in their study were stored in $CHCl_3$, and this solvent was removed only by evaporating under a stream of N_2 gas. In our experience this is inadequate to remove residual $CHCl_3$, and, as mentioned above, a minimum of 15 h under high vacuum is necessary to eliminate the strong influence of this solvent on increasing H^+/OH^- permeability. These authors also report that KOH was added during sonication to maintain constant pH, implying that acid is produced during sonication. This could be due to the production of fatty acids or to the hydrolysis of some of the residual $CHCl_3$ to produce HCl. Under any condition, caution must be exercised in interpreting the results of Clement and Gould in terms of an intrinsic H^+/OH^- permeability. Deamer (1982) has estimated a P_{NET} of 3×10^{-3} cm/s for the vesicle preparation described by Clement and Gould. This is $\sim 10^4$ times greater than that found by the present method.

Nichols et al. (1980) have studied H^+/OH^- transport across large unilamellar vesicles using 9-amino acridine as an indicator of transmembrane pH gradient. The results described by these authors differ from ours in one fundamentally important way, and that is that transmembrane pH gradients as large as 2.8 pH units relax essentially completely, and, after relaxation, the membrane potential is indirectly estimated to be near zero. This can only occur if ions other than H^+/OH^- are flowing to compensate for the charge movement, and Nichols et al. (1980) assume that Na^+ is the compensating ion. In support of this notion, they independently estimate P_{Na^+} to be 1×10^{-10} cm/s, $\sim 10^4$ times larger than that measured earlier by Hauser (1972) for sonicated vesicles, and large enough to compensate H^+/OH^- flow. This is very different from the behavior observed here. In the presence of Na^+, H^+/OH^- transport generates a full Nernstian diffusion potential in the equilibrium state, and this potential is stable for many hours, indicating the extreme impermeability of Na^+ and all other ions in the medium. If the permeability of Na^+ in the sonicated vesicles were 10^{-10} cm/s, a steady state potential much less than the Nernstial potential would be observed and would certainly not persist over the 24 h time period we have studied the system. In addition, we have shown that the number of H^+/OH^- ions transported to reach the final observed potential is consistent with the number required to charge the membrane capacitance, i.e., the only charge movement is apparently due to H^+/OH^-.

Nichols and Deamer (1980) have investigated the H^+/OH^- fluxes in large unilamellar vesicles using an acid-base titration technique and extremely small transmembrane pH gradients, thus insuring that the rate of decay of the gradient is not limited by build-up of a transmembrane potential. Using this approach, Nichols and Deamer find a net H^+/OH^- permeability of 1.4×10^{-4} cm/s at pH 7.0 with a pH gradient of 0.06 pH units. Because the permeability measured by Nichols and Deamer varies by only a factor of ~2 in the range of pH 6.5 to 7.5, this value can be directly compared with the net permeability of 5×10^{-7} cm/s obtained here for a gradient created with inside and outside pH of 6.51 and 7.55, respectively. The fairly large discrepancy in permeabilities could conceivably be related to vesicle size differences.

Gutknecht and Walter (1981) have recently studied both electroneutral HCl fluxes and electrogenic H^+/OH^- fluxes in solvent-containing planar bilayers. These authors find an electroneutral H^+ transport at high HCl concentrations (0.3 M), but no significant transport when H_2SO_4 replaced HCl. The flux follows the activity of molecular HCl and strongly supports the notion of transmembrane molecular HCl flow. In the experiments reported here, the method of measurement only detects electrogenic flux, but under any condition no Cl^- was present in media and we expect no electroneutral flux. Gutknecht and Walter (1981) have also measured H^+/OH^- conductances at 100 mM HCl in the planar bilayer system and find a range of 10^{-6} to 10^{-7} Scm^{-2}. It is not possible to compare this value to that found for vesicles in the present report since our experiments were done near 10^{-7} M H^+, and the concentration dependence of conductance has not been studied over a wide range in either system. Furthermore, it is not possible in general to compute permeability from conductance or vice versa without knowing the transport mechanism. Because the mechanism is unknown for H^+/OH^-

transport, such a calculation cannot be performed as a basis for comparing our results with those of Gutknecht and Walter.

From the above information it appears that the large unilamellar vesicles prepared by the ether injection method have a substantially higher H^+/OH^- permeability than sonicated vesicles of the same lipid. However, even in the sonicated vesicle system the H^+/OH^- permeability is anomolously high compared with other monovalent cations, and suggests a special mechanism for transport.

It would not be profitable to attempt to fit our data to a specific model for transport without more information related to the mechanism, but as concluded above, the mechanism cannot be simple passive diffusion of H^+ or OH^-. It is possible, however, that the transport is carrier mediated by a highly effective trace impurity acting as an ionophore. Fatty acids themselves could act as proton ionphores, and surely some free fatty acids must be present in any phospholipid preparation due to hydrolysis of the fatty acid glycerol esters. In our preparations, free fatty acids are not detectable by thin layer analysis even after sonication, but this does not rule out the presence of low levels of a very active species.

As shown by Fig. 7 *b*, lipid oxidation substantially increases the H^+/OH^- flux. This could occur as the result of several different effects. First, lipid oxidation will certainly increase the local membrane dielectric and enhance transmembrane ion movement simply as a result of a reduced dielectric energy of the ion in the membrane. Alternatively, the oxidized sites may act as local potential minima for the passage of a H^+ across the membrane. In this mechanism, the H^+ would simply diffuse from site to site in its transmembrane passage. Finally, oxidation may produce as products molecules capable of acting as proton carriers. Pertinent to these last two mechanisms is the observation of Toyoshima and Thompson (1975) that oxidation of egg phosphatidylcholine vesicles produces titratable proton binding sites. Clearly a species capable of binding H^+ is a candidate for a carrier or a member of a series of groups providing a transmembrane conductive pathway.

As a preliminary attempt to investigate the influence of unsaturated centers and oxidation products, we examined the H^+/OH^- permeability of vesicles formed from a saturated nonoxidizable phospholipid, diphytanoylphosphatidylcholine. The data shown in Fig. 7 *b* clearly demonstrate tremendously reduced H^+/OH^- permeability. The reduced permeability compared with egg PC could be due to any one of or a combination of several factors that include: (*a*) a larger dipole potential in the membrane, (*b*) a lower diffusion coefficient in the bilayer due to a lower fluidity than egg PC, or (*c*) the lack of unsaturated centers and/or oxidized sites. A priori, one does not expect a significant dipole potential difference between egg PC and diphytanoyl PC. Because the transport of the phosphonium in the presence of ϕ_4B^- is only 2–3 times slower than for egg PC, it appears that the diffusion coefficient of small molecules is not sufficiently reduced to explain the difference in H^+/OH^- transport. The addition of small amounts of oxidized lipid, at levels that should not significantly alter the membrane fluidity (2 mol %), dramatically increase the rate of H^+/OH^- transport in diphytanoyl lipids. Thus, point (*c*) above is a good candidate for the reduced permeability of vesicles formed from these lipids.

Although lipid oxidation products certainly contribute to H^+/OH^- conduction as discussed above, other mechanisms are possible. In particular, linear aggregates of water molecules in the bilayer interior may provide conductive pathways for protons (Nichols and Deamer, 1980). This mechanism could also give rise to a linear current-voltage characteristic as observed.

In summary, the primary purpose of this communication was to demonstrate the feasibility of measuring very small ionic currents in phospholipid vesicle systems and to clarify certain aspects of H^+/OH^- conduction in lipid bilayers. These experiments lay the foundation for application of the methods to the study of H^+ and other ionic currents in more complex protein containing membrane systems. Such work is presently underway.

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