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Measurement of Transmembrane Potentials in Phospholipid Vesicles

(spin labels/valinomycin)

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ABSTRACT Phosphatidylcholine vesicles are permeable to tempotartrate, a spin-label derivative of tartaric acid. The inside–outside distribution of tempotartrate is coupled to the inside–outside distribution of H^+, so it must be a measure of the transmembrane electrical potential difference in vesicles permeable to H^+. This prediction is borne out by the finding that the inside–outside distribution of tempotartrate is the reciprocal of the inside–outside distribution of K^+ in vesicles prepared in the presence of valinomycin.

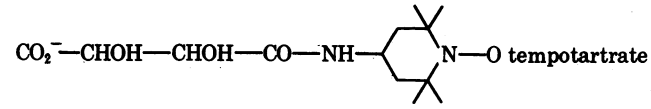
The inside–outside distribution of tempotartrate is, by contrast, equal to the inside–outside distribution of Cl^- in vesicles without valinomycin. This is evidence that an inside–outside Cl^- concentration gradient induces an H^+ gradient, which must be due to HCl permeation.

The size of microelectrodes has limited electrical measurements on membranes to large cells and extended lipid bilayers. The electrical features of most membranes are obscure.

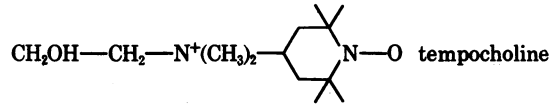
Here we report a method of measuring transmembrane potentials in small cells, cell membrane vesicles, and phospholipid vesicles. We report, in particular, on measurements of K^+ diffusion potentials in phosphatidylcholine (PChol) vesicles.

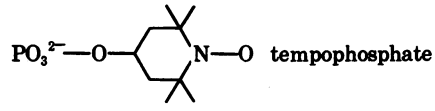
MATERIALS AND METHODS

Tempotartrate. A solution of 1.35 g of 1-oxyl-4-amino-2,2,-6,6-tetramethylpiperidine (1) and 1.35 g of tartaric acid (Brothers Chemical Co.) in 20 ml of *N,N*-dimethylformamide was mixed with a solution of 1.9 g *N,N'*-dicyclohexylcarbodiimide (Schwarz-Mann, ultra pure) in 20 ml of *N,N*-dimethylformamide. After 4 hr, 0.5 ml of glacial acetic acid was added and the mixture was stirred for 15 min. Then 200 ml of water was added and the mixture was filtered by gravity, applied to a column of 40 g of DEAE-Sephadex A-25 (Pharmacia) in 5 mM NH_4HCO_3, and eluted with a linear gradient of NH_4HCO_3 (1.6 liters, 5–100 mM). The major yellow band, which emerged from the column toward the end of the gradient, was evaporated under reduced pressure. The residual solids were dispersed in absolute ethanol and filtered by gravity, and the filtrate was evaporated under reduced pressure. The residual solids were dispersed in $CHCl_3$ and filtered by suction through sintered glass, and the filtrate was evaporated under reduced pressure. Paper chromatography of the orange solid product (Whatman 3 MM paper, developed descending for 6 hr in absolute ethanol–1 M NH_4OAc in water 5:2) revealed two spots (both UV-absorbing and both stained by iodine vapor; R_F values 0.7 and 0.9). The spot of R_F value 0.7 was about 10 times as intense as the other spot. Spots of R_F value 0.7 from several preparative chromatograms were eluted with water and evaporated under reduced pressure. The residual solids were dispersed in acetone and centrifuged, and the supernatant was evaporated under reduced pressure. The orange solid product was converted to the Na^+ salt by passage through the Na^+ form of AG 50W-X8 (BIO-RAD Laboratories) in water. Infrared (KBr) bands were observed at 1655 cm^{-1} (carboxylic amide) and 1610 and 1380 cm^{-1} (carboxylate).



Miscellaneous Materials. Tempocholine was prepared as described (2). Tempophosphate was prepared by the procedure of Weiner (3), and further purified by chromatography on DEAE-Sephadex A-25 (30 g) with a linear gradient of NH_4HCO_3 (1 liter, 0.01–0.2 M). *p*-Trifluoromethoxycarbonylcyanide phenylhydrazone (FCCP) was a gift of Dr. P. G. Heytler, E. I. Du Pont De Nemours and Co., Wilmington, Del.





Procedures. The preparation of egg PChol vesicles and measurements of paramagnetic resonance were as described (ref. 4 and 2, respectively). The concentration of phospholipid phosphate was 50 mM; 50 μl of ice-cold vesicle solution was mixed with 5 μl of ice-cold 0.33 M sodium ascorbate (pH 7); and the amplitudes of paramagnetic resonance spectra were measured on the low-field lines.

Treatment of Data. The inside–outside tempotartrate distributions in Table 3 and Fig. 1 (the values of $[T^-]_i/[T^-]_o$) were determined from the amplitudes of paramagnetic resonance spectra of mixtures of vesicles with ascorbate by (*a*)

Abbreviations: PChol, phosphatidylcholine; FCCP, *p*-trifluoromethoxycarbonylcyanide phenylhydrazone; tempo, 2,2,6,6-tetramethylpiperidine-1-oxyl.

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multiplication by the factor of dilution at step *ii* in the experiment (see *Results*) to normalize all amplitudes to the same vesicle concentration, (*b*) division by the amplitude of the paramagnetic resonance spectrum of the vesicle solution without ascorbate, to correct for loss of spin-label paramagnetism during sonication (there was always about 40% loss of spin-label paramagnetism from egg PChol vesicle preparations containing tempotartrate during sonication), (*c*) subtraction, from all amplitudes alike, of the difference of amplitudes between vesicles kept at 0° and vesicles kept at 30° without dilution at step *ii* in the experiment (see *Results*) (the amplitudes of paramagnetic resonance spectra of mixtures of aliquots of these vesicles with ascorbate were measured after various times; the amplitude for the vesicles at 0° was constant, whereas the amplitude for the vesicles at 30° increased by about 30%), and (*d*) correction for the spin-exchange interaction of tempotartrate inside the vesicles by means of relations we obtained from measurements of amplitudes of paramagnetic resonance spectra of 1–10 mM sodium tempotartrate in water at 0°:

$$\text{tempotartrate concentration (mM)} = A/10, \qquad A < 30$$

$$\text{tempotartrate concentration (mM)} = 0.130 + 0.0777\,A + 0.00053\,A^2, \qquad A \geq 30$$

A is the amplitude of the paramagnetic resonance spectrum.

For the treatment of data in Fig. 4, step *a* above was omitted, and the difference of amplitudes in step *c* above was taken from measurements on vesicles diluted with 2 volumes of the solution from which the vesicles were prepared.

For the treatment of data in Tables 2 and 4, the factors of dilution in step *a* above were obtained by phosphate determinations (5) on the Sephadex G-25 eluates, the division in step *b* above was by the difference between the amplitude after step *a* above and the amplitude for the vesicle solution without ascorbate (to correct for the decrease in the concentration of tempotartrate outside the vesicles), and the difference of amplitudes in step *c* was for Table 2, 10% of the average of the three values from the experiments of Fig. 1 (10% because the tempotartrate concentration was 0.1 times as great in the experiments of Table 2 as in the experiments of Fig. 1), and for Table 4, 10% of the average of the two values from the experiments of Fig. 4.

TABLE 1. *PChol vesicles are permeable to tempotartrate and impermeable to tempocholine and tempophosphate*

	Spin label inside of vesicles as % of total spin label	
	Spin label added before sonication	Spin label added after sonication and allowed to equilibrate for 4 hr at 30°
Tempotartrate	0.81	0.78
Tempocholine	0.82	0.10
Tempophosphate	0.76	0.10

In the experiments in which spin label was added before sonication, the vesicles were prepared from 0.2 M NaCl–0.05 M sodium phosphate (pH 7.0)–2.5 mM spin label. In the experiments in which spin label was added after sonication, the vesicles were prepared from 0.2 M NaCl–0.05 M sodium phosphate (pH 7.0), and 0.4 ml of the vesicle preparation was mixed with 0.01 ml of 0.1 M spin label.

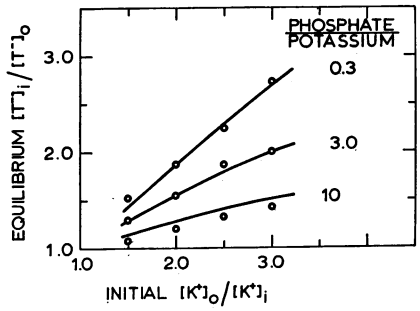

FIG. 1. The equilibrium value of the tempotartrate distribution depends on buffer and K^+ concentrations in a predictable way. *Circles* are results of experiments, *curves* are results of calculations (*Methods*). The procedure was as outlined in the text (steps *i–iv*). The values of phosphate/potassium are ratios of phosphate buffer to K^+ concentrations in the solutions from which the vesicles were prepared (step *i*); the value of 0.3 corresponds to a solution of 0.3 M Na^+–0.1 M K^+–0.03 M phosphate (pH 6.3)–0.18 M SO_4^{2-}–2.5 mM sodium tempotartrate, the value of 3.0 corresponds to a solution of 0.15 M Na^+–0.05 M K^+–0.15 M phosphate (pH 6.3)–2.5 mM sodium tempotartrate, and the value of 10 corresponds to a solution of 0.37 M Na^+–0.03 M K^+–0.28 M phosphate (pH 6.3)–2.5 mM sodium tempotartrate. Valinomycin (Calbiochem) was mixed with the egg PChol (1.5 μg valinomycin/μmol phospholipid phosphate) before the preparation of vesicles was begun. The vesicles were diluted with 0.5, 1, 1.5, and 2 volumes in step *ii* of the experiment. The vesicles of phosphate/potassium values 0.3, 3.0, and 10 were diluted with 0.4 M K^+–0.03 M phosphate (pH 6.3)–0.18 M SO_4^{2-}–2.5 mM sodium tempotartrate, 0.2 M K^+–0.15 M phosphate (pH 6.3)–2.5 mM sodium tempotartrate, and 0.39 M K^+–0.29 M phosphate (pH 6.3)–2.5 mM sodium tempotartrate, respectively. In step *iii* of the experiment, the vesicles were kept for 3.5 hr at 30° with no further addition of tempotartrate.

Calculations of Equilibrium Ion Distributions. The calculations for Tables 2 and 3 and Fig. 1 are based on the following equations.‡

$$\frac{[K^+]_o^e}{[K^+]_i^e} = \frac{[H^+]_o^e}{[H^+]_i^e} = \frac{[T^-]_i^e}{[T^-]_o^e} \equiv \frac{1}{x} \qquad (1)$$

$$[K^+]_i^i + [H^+]_i^i + [Na^+]_i^i - 2[HPO_4^{2-}]_i^i - [H_2PO_4^-]_i^i - [T^-]_i^i - 2[SO_4^{2-}]_i^i = 0 \qquad (2)$$

‡ The term $-3.2 \times 10^{-4} \log_e x$ is the concentration inside a vesicle of the ions that traverse the membrane to produce the transmembrane electrical potential difference. It was calculated from the expression

$$-(V/0.0768\,Lv)$$

where V is given by Eq. 1 and 9, 0.0768 is the transmembrane electrical potential difference (in volts) that results from one ion traversing the membrane (based on an inner vesicle radius of 75 Å, an outer vesicle radius of 125 Å, and a membrane dielectric constant of 1), L is Avogadro's number, and v is the volume of a vesicle.

TABLE 2. *Tempotartrate measures a valinomycin-induced K^+ diffusion potential in PChol vesicles*

Valinomycin per vesicle	Initial $[K^+]_o/[K^+]_i$	Equilibrium $[K^+]_o/[K^+]_i$	Equilibrium $[T^-]_i/[T^-]_o$ Expected	Observed
0	10	10	1.0	1.3
0.2	10	6.6	6.6	6.2
0	20	20	1.0	1.4
0.2	20	12.0	12.0	9.8

The procedure was as outlined in the *text* (steps *i–iv*). The vesicles, with valinomycin (as described in Fig. 1, except 0.06 μg valinomycin/μmol phospholipid phosphate) or without valinomycin, were prepared from 0.475 M Na^+–0.025 M K^+–0.025 M phosphate (pH 6.3)–0.23 M SO_4^{2-} and passed through a column of Sephadex G-25 fine (Pharmacia) into 0.25 M Na^+–0.25 M K^+–0.025 M phosphate (pH 6.3)–0.23 M SO_4^{2-} (for an initial value of $[K^+]_o/[K^+]_i$ of 10) or 0.5 M K^+–0.025 M phosphate (pH 6.3)–0.23 M SO_4^{2-} (for an initial value of $[K^+]_o/[K^+]_i$ of 20) at about 2°. The diameter and void volume of the column were 11 mm and 7 ml. The Sephadex eluate (0.2 ml) was mixed with 5 mM tempotartrate in column buffer (0.01 ml) and kept for 3.5 hr at 30°. The method of calculation of equilibrium values of $[K^+]_o/[K^+]_i$ for vesicles with valinomycin is outlined in *Methods*.

$$[K^+]_i^e + [H^+]_i^e + [Na^+]_i^e - 2[HPO_4^{2-}]_i^e - [H_2PO_4^-]_i^e - [T^-]_i^e - 2[SO_4^{2-}]_i^e = -3.2 \times 10^{-4} \log_e x \quad (3)$$

$$[HPO_4^{2-}]_i + [H_2PO_4^-]_i = C \quad (4)$$

$$\frac{[HPO_4^{2-}]_i[H^+]_i}{[H_2PO_4^-]_i} = K \quad (5)$$

The subscripts i and o denote inside and outside, the superscripts i and e denote initial and equilibrium, T^- denotes tempotartrate, C is the concentration of phosphate buffer, and $K \equiv K' \gamma^{H_2PO_4^-}/\gamma^{HPO_4^{2-}}\gamma^{H^+}$, where K' is the dissociation constant of $H_2PO_4^-$ and γ denotes an activity coefficient (in the experiments of Tables 2 and 3 and Fig. 1, the ionic strengths of the solutions inside and outside the vesicles were very nearly equal, so we have taken the activity coefficients of the ions inside and outside the vesicles to be equal). The method of calculation for Table 4 and Fig. 4 is the same, except that Eq. 1 is replaced by

$$\frac{\gamma_i^{Cl^-}[Cl^-]_i^e}{\gamma_o^{Cl^-}[Cl^-]_o^e} = \frac{\gamma_o^{H^+}[H^+]_o^e}{\gamma_i^{H^+}[H^+]_i^e} = \frac{\gamma_i^{T^-}[T^-]_i^e}{\gamma_o^{T^-}[T^-]_o^e} \equiv \frac{1}{x}$$

(in the experiments of Table 4 and Fig. 4, the ionic strengths of the solutions inside and outside the vesicles were very different, so we could not take the activity coefficients of the ions inside and outside the vesicles to be equal), $[K^+]_i$ in Eq. 2 and 3 is replaced by $-[Cl^-]_i$, the term $-3.2 \times 10^{-4} \log_e x$ in Eq. 3 is omitted, and the activity coefficients in the definition of K are for ions inside the vesicles. We have solved these equations for x in terms of the initial conditions, taking the concentrations of all ions outside the vesicles to be constant [because the volumes of the aqueous interiors of the vesicles were less than 1% of the total volumes of our vesicle preparations (see Table 1 and related text)], taking $[Na^+]_i^i = [Na^+]_i^e$ and $[SO_4^{2-}]_i^i = [SO_4^{2-}]_i^e$, and making use of the Debye-Hückel formula (6) for the activity coefficients [with values of $10^8 a_i$ (see ref. 6) of 3 for K^+, 9 for H^+, 3.5 for T^-, and 4 for HPO_4^{2-} and $H_2PO_4^-$].

RESULTS

PChol vesicles are permeable to tempotartrate

Throughout this work, we have used the PChol vesicles that result from prolonged sonication of egg PChol in salt solution. Each vesicle has a bilayer membrane of 125-Å hydrodynamic radius (2, 7, 8). In previous work, we found the membrane to be impermeable to ascorbate at 0° (2). This finding, together with the fact that ascorbate abolishes the paramagnetism of nitroxide spin labels, enables us to observe the paramagnetic resonance of spin labels inside egg PChol vesicles. In the experiments of the first column of Table 1, for example, we included an ionic spin label in the solution from which the vesicles were prepared and recorded the paramagnetic resonance spectrum of a mixture of ascorbate with the vesicle preparation at 0°; we took the amplitude of the spectrum as a measure of the quantity of spin label in the aqueous interiors of the vesicles. The results attest in two ways to the validity of the procedure. First the results are the same for three different spin labels, tempotartrate (a spin-label derivative of tartaric acid), tempocholine (a spin-label derivative of choline), and tempophosphate (a spin-labeled phosphate ester). This consistency argues against such possible artifacts as a specific association of spin label with the vesicle membranes. Second, the results are compatible with the contained volumes we previously found for PChol vesicles by the use of radioactive disaccharides and gel chromatography [the result for tempotartrate in Table 1 corresponds to an average contained volume for all the egg PChol vesicles in the preparation of 0.16 μl/μmol of phosphate; we previously found contained volumes for disaccharides in a spin-labeled PChol–egg PChol vesicle (5:95, mol %) preparation that ranged from 0.10 to 1.0 μl/μmol of phosphate, with an average of 0.13 μl/μmol of phosphate (2)].

We use these results to determine the permeabilities of egg PChol vesicles to tempotartrate, tempocholine, and tempophosphate. We mixed the vesicles with spin label at 30° and waited 4 hr, then chilled the mixture to 0°, added ascorbate, and recorded the paramagnetic resonance spectrum. We found (second column of Table 1) that at 30° egg PChol vesicles are permeable to tempotartrate and relatively impermeable to tempocholine and tempophosphate. The difference in permeabilities must be due to the relatively much greater concentration of tempotartrate in its uncharged form (which we denote by HT). Evidence that tempotartrate does penetrate PChol vesicles in its uncharged form is presented

TABLE 3. *Valinomycin-induced K^+ diffusion potentials in PChol vesicles can be made inside negative*

Initial $[K^+]_o/[K^+]_i$	Equilibrium $[T^-]_i/[T^-]_o$ Expected	Observed
0.67	0.67	0.72
0.40	0.41	0.39

The procedure was as described in Fig. 1, with the following exceptions. The vesicles were prepared from 0.18 M K_2SO_4–0.03 M potassium phosphate (pH 6.5)–2.5 mM sodium tempotartrate, and diluted with 0.5 and 1.5 volumes of 0.18 M Na_2SO_4–0.03 M sodium phosphate (pH 6.5)–2.5 mM sodium tempotartrate. The method of calculation of the expected equilibrium values of $[T^-]_i/[T^-]_o$ is outlined in *Methods*.

below. A consequence of this fact is that the equilibrium concentration of tempotartrate in its uncharged form must be the same on the inside and outside of PChol vesicles ($[HT]_i = [HT]_o$). Taking into account also the dissociation equilibrium of tempotartrate ($[H^+]_i[T^-]_i/[HT]_i = K_d$ and $[H^+]_o[T^-]_o/[HT]_o = K_d$), we find

$$\frac{[T^-]_i}{[T^-]_o} = \frac{[H^+]_o}{[H^+]_i} \qquad (6)$$

That is, the inside–outside distribution of tempotartrate is coupled to the inside–outside distribution of H^+.

Tempotartrate measures a valinomycin-induced K^+ diffusion potential in PChol vesicles

The permeability of PChol vesicles to H^+ is not known, but it can almost certainly be enhanced by the addition of an H^+ carrier such as FCCP. Whatever the mechanism, if PChol vesicles are permeable to H^+, then the inside–outside distribution of H^+ must reflect a transmembrane electrical potential difference. Specifically,

$$\frac{[H^+]_o}{[H^+]_i} = \exp(-eV/kT) \qquad (7)$$

where e is the fundamental unit of charge, V is the transmembrane electrical potential difference, and k is Boltzmann's constant. Eq. 6 and 7 imply that the inside–outside distribution of tempotartrate should also reflect a transmembrane electrical potential difference:

$$\frac{[T^-]_i}{[T^-]_o} = \exp(-eV/kT) \qquad (8)$$

We tested these predictions as follows. (*i*) We prepared egg PChol vesicles, with a small admixture of the antibiotic molecule valinomycin, from solutions of high Na^+ and low K^+ concentrations. (*ii*) We passed the vesicles, by dilution

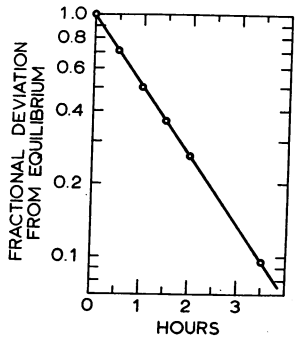

FIG. 2. The approach to equilibrium of the inside–outside tempotartrate distribution is a first-order process. The vesicles were prepared from 0.2 M NaCl–0.05 M sodium phosphate (pH 7.0)–2.5 mM sodium tempotartrate, diluted with 2 volumes of 0.4 M sucrose–0.05 M sodium phosphate (pH 7.0)–2.5 mM sodium tempotartrate, and kept at 30°. After various times, t, the amplitudes of paramagnetic spectra of mixtures of aliquots of the vesicle solution with ascorbate were measured. These amplitudes were corrected for the spin-exchange interaction of tempotartrate inside the vesicles, according to step *d* of the procedure in *Methods*, then designated $A(t)$, and used to compute $[A(\infty) - A(t)]/[A(\infty) - A(0)]$, which was designated as the fractional deviation from equilibrium.

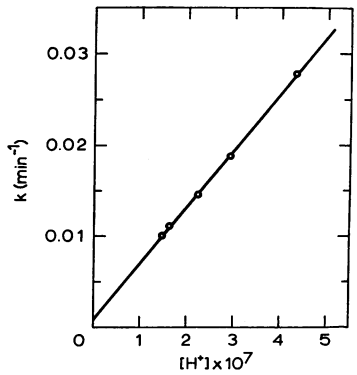

FIG. 3. The rate of approach to equilibrium of the tempotartrate distribution is proportional to the H^+ concentration. The values of k are the slopes of plots like the one in Fig. 2. They correspond to vesicle preparations at pH values of 6.91, 6.87, 6.73, 6.61, and 6.44. [The hydrogen-ion activity coefficient was taken to be 0.84 (6).] The vesicles were prepared from 0.25 M NaCl–0.025 M sodium phosphate–2.5 mM sodium tempotartrate, diluted with 2 volumes of 0.4 M sucrose–0.025 M sodium phosphate–2.5 mM sodium tempotartrate, and kept at 30°.

or gel filtration, into solutions of high K^+ and low Na^+ concentrations. Since valinomycin makes PChol vesicles selectively permeable to K^+ (9), there must be a flux of K^+ into the vesicles that creates a transmembrane electrical potential difference and continues until

$$\frac{[K^+]_o}{[K^+]_i} = \exp(-eV/kT) \qquad (9)$$

(*iii*) We added some tempotartrate and allowed its inside–outside distribution to come to equilibrium. (*iv*) We chilled the mixture to 0°, added ascorbate, and recorded the paramagnetic resonance spectrum. The results are shown in the last column of Table 2. They conform to Eq. 8 and 9 in that for vesicles without valinomycin Eq. 8 requires $[T^-]_i/[T^-]_o = 1$ (vesicles without valinomycin should develop no transmembrane electrical potential difference), and for vesicles with valinomycin Eq. 8 and 9 imply $[T^-]_i/[T^-]_o = [K^+]_o/[K^+]_i$.

The results have two implications for the properties of valinomycin. The first concerns the valinomycin-vesicle stoichiometry. One valinomycin per five vesicles is sufficient to induce transmembrane electrical potential differences in all the vesicles, a result consistent with the fact (9) that one valinomycin per 30 vesicles is sufficient to make all the vesicles permeable to K^+. Valinomycin can evidently pass from one vesicle to another.

The second implication concerns the permeability of the vesicles to H^+. The results for vesicles with valinomycin are not affected by the addition of an H^+ carrier (1 ml of vesicle solution was mixed with 0.01 ml of 1 mM FCCP in methanol at step *ii* above), so either the vesicles with valinomycin are permeable to H^+ or the tempotartrate and K^+ distributions are not coupled to the H^+ distribution. We think the vesicles with valinomycin are permeable to H^+, in view of the following two lines of evidence for the coupling of the ion distributions.

The first line of evidence concerns the coupling of the tempotartrate and H^+ distributions. We measured the increase with time of the quantity of tempotartrate inside the

TABLE 4. *Tempotartrate measures an inside–outside Cl^- concentration gradient in PChol vesicles*

Initial $[Cl^-]_i/[Cl^-]_o$	Equilibrium $[Cl^-]_i/[Cl^-]_o$ (expected)	Equilibrium $[T^-]_i/[T^-]_o$ (observed)
5.5	5.0	4.3
10.5	9.3	9.4
20.5	17.7	14.1

The procedure was as described in Table 2, with the following exceptions. The vesicles were prepared from 0.15 M NaCl–0.03 M sodium phosphate (pH 6.5) without valinomycin. The column buffers were mixtures of 0.15 M NaCl–0.03 M sodium phosphate (pH 6.5) and 0.26 M sucrose–0.03 M sodium phosphate (pH 6.5). The method of calculation of the expected equilibrium values of $[Cl^-]_i/[Cl^-]_o$ is outlined in *Methods*.

vesicles at step *iii* above; that is, we measured the rate of approach to equilibrium of the tempotartrate distribution. We found half-times of 17 min at pH 6.34 and 30° and 63 min at pH 6.96 and 30°. These half-times are consistent with the results of a more detailed rate–pH study (see below, Figs. 2 and 3). Evidently the rate of approach to equilibrium of the tempotartrate distribution is proportional to the H^+ concentration. This is a necessary condition for permeation of the vesicles by tempotartrate in its uncharged form, which is in turn a sufficient condition for Eq. **6**.

The second line of evidence concerns the coupling of the K^+ and H^+ distributions. We measured the effect on the K^+ distribution of an H^+ buffer in the vesicle preparation. This can be understood qualitatively as follows. The flux of K^+ into the vesicles at step *ii* above makes the interiors of the vesicles electrically positive. This causes an H^+ efflux, which tends to restore the vesicles to electrical neutrality. The cycle (K^+ influx, H^+ efflux) repeats itself until $[H^+]_o/[H^+]_i = [K^+]_o/[K^+]_i$ (Eq. **7** and **9**).§ The number of ions of K^+ and H^+ that must move to produce this equilibrium condition are made larger by the presence of an H^+ buffer in the vesicle preparation (since the number of H^+ ions that must move to alter $[H^+]_o/[H^+]_i$ is made larger by the presence of an H^+ buffer). The equilibrium value of $[K^+]_o/[K^+]_i$ is diminished by the K^+ movement, so it is diminished by the presence of an H^+ buffer. An exact calculation of the dependence of the equilibrium value of $[K^+]_o/[K^+]_i$ on the concentration of the H^+ buffer is given in *Methods*. This calculation is compared with the results of experiments in Fig. 1.

In the experiments of Table 2 and Fig. 1, the transmembrane electrical potential difference had the same sign ($[K^+]_o/[K^+]_i > 1$, inside positive). We have done further experiments to check that the transmembrane electrical potential difference can be made to have the opposite sign ($[K^+]_o/[K^+]_i < 1$, inside negative). The results are shown in Table 3.

An inside–outside Cl^- concentration gradient induces an H^+ gradient in PChol vesicles

We have found that the tempotartrate distribution does not reflect the K^+ distribution in vesicles without an ion carrier (Table 2). We find by contrast that the tempotartrate distribution does reflect the Cl^- distribution in vesicles without an ion carrier. Specifically, we find (Table 4) that

$$\frac{[T^-]_i}{[T^-]_o} = \frac{[Cl^-]_i}{[Cl^-]_o} \qquad (10)$$

at equilibrium.

We have presented the results of rate–pH and buffer-dependence studies on vesicles with valinomycin (see above) as evidence of the coupling of the tempotartrate and K^+ distributions by the H^+ distribution. We present the results of analogous experiments as evidence of the coupling of the tempotartrate and Cl^- distributions by the H^+ distribution. The results of the rate–pH study are shown in Figs. 2 and 3. The pseudo first-order rate constant for tempotartrate equilibration is evidently proportional to the H^+ concentration. This is a necessary condition for permeation of the vesicles by tempotartrate in its uncharged form, which is in turn a sufficient condition for Eq. **6**. Eqs. **6** and **10** imply

$$\frac{[H^+]_o}{[H^+]_i} = \frac{[Cl^-]_i}{[Cl^-]_o} \qquad (11)$$

That is, an inside–outside Cl^- concentration gradient induces an H^+ gradient.

The results of the buffer-dependence study are shown in Fig. 4. The calculated curves are based on a 1:1 stoichiometry of Cl^- and H^+ movements¶. This stoichiometry could be due to either Cl^- and H^+ permeation or HCl permeation [in the case of Cl^- and H^+ permeation, the coupling of the Cl^- and H^+ distributions would be electrical, the stoichiometry would be due to a cycle of Cl^- efflux and H^+ efflux (analogous to the cycle of K^+ influx and H^+ efflux in vesicles with valinomycin), and Eq. **11** would follow from $[Cl^-]_i/[Cl^-]_o = \exp(-eV/kT)$ and Eq. **7**; in the case of HCl permeation, which Bangham has suggested to account for isotopic Cl^- fluxes (10), the coupling of the Cl^- and H^+ distributions would be analogous to the coupling of the tempotartrate and H^+ distributions, and the justification for Eq. **11** would be analogous to the justification for Eq. **6**]. Cl^- and H^+ permeation cannot account for all the results, unless the Cl^- and H^+ permeation rates are at least an order of magnitude greater than the Na^+ permeation rate. Electrical measurements on extended phosphatidylcholine and phosphatidylethanolamine bilayers have indicated that the Cl^-, H^+, and Na^+ permeation rates are of the same order of magnitude (11, 12). So, there must be HCl permeation.

DISCUSSION

We set out to find a spin-labeled ion ($X^\pm$) that traverses the membranes of PChol vesicles in a charged form. We could then calculate the transmembrane electrical potential difference from the equation

$$\frac{[X^\pm]_i}{[X^\pm]_o} = \exp(\pm eV/kT) \qquad (12)$$

We were unsuccessful in this approach. The best we could do was to find a spin-labeled ion that traverses the membranes of PChol vesicles in an uncharged form (tempotartrate). Eq. **12** is not valid in general when $X^\pm$ traverses membranes in an

§ This is not fundamentally different from the interpretation by Mitchell and Moyle (14) of the effect of valinomycin and FCCP on the kinetics of acid-base titrations of mitochondria.

¶ A 1:1 stoichiometry of Cl^- and H^+ movements is equivalent to Eq. **2–4** (with $-[Cl^-]_i$ replacing $[K^+]_i$ and the term $-3.2 \times 10^{-4} \log_e x$ being omitted) because $[H^+]_i$ and $[T^-]_i$ are negligible in comparison to the other terms.

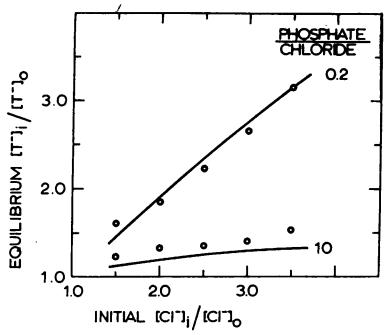

FIG. 4. The equilibrium value of the tempotartrate distribution depends on buffer and Cl^- concentrations in a predictable way. *Circles* are results of experiments, *curves* are results of calculations (*Methods*). The procedure was as described in Fig. 1, with the following exceptions. The values of phosphate/chloride are ratios of phosphate buffer to Cl^- concentrations in the solutions from which the vesicles were prepared; the value of 0.2 corresponds to a solution of 0.15 M NaCl–0.03 M sodium phosphate (pH 6.5)–2.5 mM sodium tempotartrate, and the value of 10 corresponds to a solution of 0.03 M NaCl– 0.3 M sodium phosphate (pH 6.3)–2.5 mM sodium tempophosphate. There was no valinomycin in the vesicle preparations. The vesicles were diluted with 2 volumes of mixtures of the solutions from which they were prepared and 0.26 M sucrose–0.03 M sodium phosphate (pH 6.5)–2.5 mM sodium tempotartrate (for the vesicles of phosphate/chloride value 0.2) or 0.26 M sucrose–0.3 M sodium phosphate (pH 6.3)–2.5 mM sodium tempotartrate (for the vesicles of phosphate/chloride value 10) to obtain the indicated initial values of $[Cl^-]_i/[Cl^-]_o$.

uncharged form. But our reasoning and results suggest that it is valid for the special case of tempotartrate in PChol vesicles permeable to H^+.

Our lack of success in finding a spin-labeled ion that traverses the membranes of PChol vesicles in a charged form must be a reflection of the impermeability of phospholipid membranes to ions in general. It is possible, however, that some biological membranes are permeable to the charged forms of spin-labeled ions. Acetylcholinesterase-rich vesicles of electroplax membrane (13), for example, are permeable to tempocholine (M. G. McNamee and H. M. McConnell, unpublished).

The largest values of transmembrane electrical potential differences in our experiments were 48 and 59 mV (Table 2 and Eq. 8). These values require only a very small separation of charge: they correspond to 0.62 and 0.77 excess monovalent cations inside each vesicle.‡

There are many possible applications of tempotartrate, or tempotartrate together with an H^+ carrier, in transmembrane potential measurements. There remain to be determined, for example, the roles of H^+ concentration gradients and transmembrane potentials in the coupling of oxidation to phosphorylation in mitochondria, the electrical features of purified fragments of electrically active membranes, and the properties of ion-gate and ion-transport proteins in phospholipid vesicles.

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