Chloride Flux in Bilayer Membranes: Chloride Permeability in Aqueous Dispersions of Single-Walled, Bilayer Vesicles†

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ABSTRACT: Aqueous dispersions of phosphatidylcholine vesicles were utilized to determine bilayer permeability to ^{36}Cl as a function of pH and temperature. These dispersions were comprised of single-walled vesicles, homogeneous in size, prepared by sonication of purified egg phosphatidylcholine under argon followed by fractionation on a molecular sieve. Permeability constants calculated from the inward flux of ^{36}Cl and the geometric parameters of these vesicles proved to be dependent on both pH and temperature. Analysis of these dependences leads to the conclusion that ^{36}Cl permeation in the presence of KCl is due principally to a carrier mediated exchange process involving a phospholipid–HCl complex. Net permeation by $H^{36}Cl$ may make a small contribution to the ^{36}Cl flux; however, studies carried out at very low chloride concentrations show that this flux is much smaller than the exchange flux. Thus chloride permeability for the exchange process is 1.5×10^{-11} cm sec^{-1} while the corresponding coefficient for the net flux of $H^{36}Cl$ is 1.0×10^{-12} cm sec^{-1} at pH 7. The activation energy for the ^{36}Cl exchange flux was found to be 19 ± 2 kcal/mol. This value is similar to that obtained for the transbilayer "flip-flop" of phosphatidylcholine molecules in a similar system (Kornberg and McConnell, 1971). This correspondence together with the fact that the experimentally determined flux of ^{36}Cl agrees well with that calculated from the "flip-flop" parameters, strongly suggests that the flux of ^{36}Cl and "flip-flop" of phosphatidylcholine may be the same process.

In the preceding paper it was reported that the high resistance semispherical bilayer membranes formed from a decane solution of synthetic diphytanoylphosphatidylcholine displayed an electrically silent ^{36}Cl flux about 10^3-fold larger than the Cl^- current calculated from the electrical parameters of the system (Toyoshima and Thompson, 1975). These permeability properties were qualitatively similar in all respects to those reported in a previous publication from this laboratory for low resistance bilayers formed from a chloroform–methanol solution of tetradecane and egg phosphatidylcholine (Pagano and Thompson, 1968). The results obtained with both types of bilayers strongly suggest that the electrically silent flux of ^{36}Cl is primarily a carrier mediated diffusion process in which bilayer phosphatidylcholine acts as the carrier species.

Recently Kornberg and McConnell (1971) have suggested that the electrically silent chloride flux may be associated with the transbilayer "flip-flop" of phosphatidycholine observed by these workers in vesicle systems using electron spin resonance techniques. The studies on single-walled vesicles reported in this paper were undertaken in order to investigate further the involvement of H^+ in the electrically silent ^{36}Cl permeation process, and to provide data for a comparison of the experimentally determined ^{36}Cl flux in this system with that calculated from the transmembrane "flip-flop" parameters determined by Kornberg and McConnell (1971). Although Bangham (1972) has suggested that the entire flux of ^{36}Cl across bilayers is due to the permeation of molecular $H^{36}Cl$, the results reported here indicate that permeation of $H^{36}Cl$ can at most make only a minor contribution to the observed ^{36}Cl flux (Toyoshima and Thompson, 1973). A similar conclusion has recently been reported by Singer (1973) based on studies carried out on multilamellar liposomes.

Experimental Section

Preparation of Phosphatidylcholine Vesicles. Phosphatidylcholine was isolated from hen egg yolk by chromatographic procedures previously described (Huang, 1969). The purity of the preparation was monitored by thin-layer chromatography (Skipski et al., 1964). Vesicle dispersions were prepared by suspending lyophilized phosphatidylcholine (400–500 mg) in 12 ml of aqueous solution of the appropriate composition (Huang, 1969). The suspension was then ultrasonically irradiated with a Branson Sonicator at 20 kHz and 4° under argon for 2 hr. Titanium fragments released from the sonication probe and undispersed phospholipid were removed by centrifugation at 70,000*g* for 10 min at 4°. The resulting supernatant was subjected to molecular sieve chromatography at 4° on a Sepharose 4B column (2.5 × 50 cm). The molecular sieve elution profile of the dispersion consisted of two distinct peaks, fractions I and II. Those portions of fraction II which showed a linear relation between absorbance at 300 nm and lipid phosphorus content were utilized in all studies (Huang and Charlton, 1972). Phosphatidylcholine concentrations in the vesicle solutions are expressed in terms of mol of lipid phosphorus/ml of P_i as determined by the Gomori (1942) modification of the Bartlett method.

Isotopic Chloride Flux Measurements. The inward directed flux of ^{36}Cl across the bilayer wall of homogeneous phosphatidylcholine vesicles was determined in two different types of systems.

(1) In the first type the composition of the ambient aqueous phase outside the vesicles was identical with that in the aqueous compartment inside each vesicle. Compositional identity of the two aqueous compartments was achieved by simply carrying out vesicle preparation in the appropriate aqueous solution.

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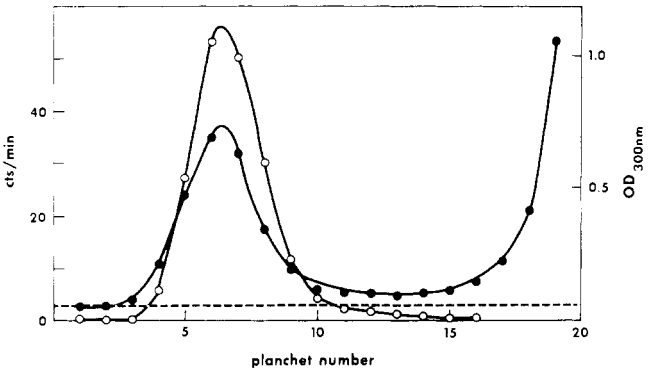

FIGURE 1: Sephadex G-50 column elution profiles of ^{36}Cl and phosphatidylcholine. (●) ^{36}Cl cpm; (○) absorption of phosphatidylcholine vesicles at 300 nm.

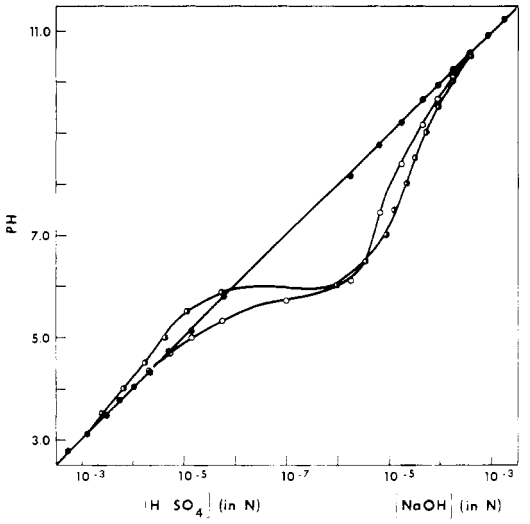

FIGURE 2: Hydrogen ion titration curves obtained by addition of H_2SO_4 or KOH. (●) Pure water, (○) 5 μmol/ml of egg phosphatidylcholine vesicle dispersion containing no electrolyte, (◑) 5 μmol/ml of egg phosphatidylcholine vesicle dispersion containing 0.5 *M* KCl.

(2) In the second type of system the composition of the external aqueous phase was different from that in the aqueous compartment inside each vesicle. In order to prepare this type of system, sonication was carried out in an aqueous phase whose composition was that required ultimately for the internal aqueous phase. Adjustment of the external phase to the desired composition was accomplished by rapid passage of the vesicle dispersion over a Sephadex G-50 molecular sieve column (2.5 × 25 cm) at 4° which had been previously equilibrated with the desired aqueous phase. Under the conditions used, the exchange of components between internal and external aqueous compartments during passage over the sieve column was negligible.

To determine the chloride flux, 0.30 ml of $K^{36}Cl$ solution (25 Ci/ml) was added to 10.0 ml of isotope-free vesicle dispersion at zero time. The dispersion was then incubated with vigorous stirring, under argon at constant temperature. At 10-min intervals 0.40-ml aliquots were removed and applied to a Sephadex G-50 molecular sieve column (1.0 × 7.0 cm) at 4° and eluted with the appropriate aqueous solution. The column eluent was collected in 5-drop fractions directly onto counting planchets. The fractions were then dried, and counted on a low background Nuclear Chicago Counter (Model 4334). The elution profile obtained in this manner showed two distinct, well-separated peaks of ^{36}Cl. The first

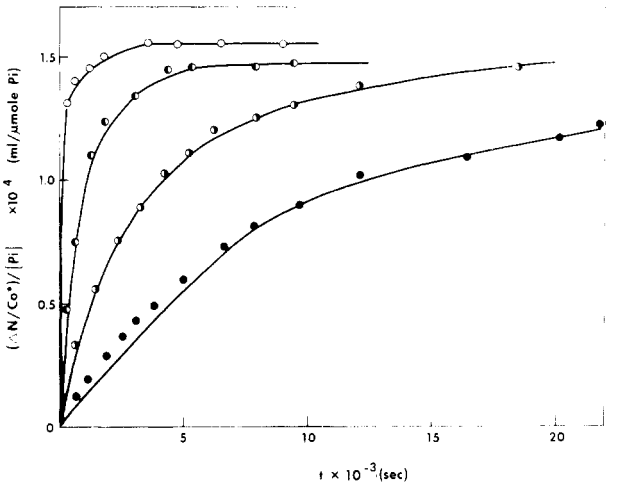

FIGURE 3: The ^{36}Cl flux at 6.3° as a function of pH. Aqueous phase 1.0 *M* KCl plus: (○) 0.1 *M* potassium acetate buffer (pH 5.0), (◐) 0.1 *M* potassium citrate buffer pH 6.0, (◑) 0.1 *M* Tris-chloride buffer (pH 7.2), (●) 0.1 *M* Tris-chloride buffer (pH 8.0). (P_i) = 10 μmol/ml.

peak, which appeared in the void volume, was coincident with the phospholipid vesicle peak as shown in Figure 1. The second peak of ^{36}Cl, which was always completely separated from the first peak, was due to the isotope present in the external aqueous phase. The small dimensions of the sieve column made it possible to complete the chromatography in about 2 min. In this short time interval, release of isotope from within the vesicle during column separation was negligible. The total flux of ^{36}Cl was taken to be the sum of the counts in planchets 3 through 12, minus the background count for each fraction.

Hydrogen Ion Titration of Vesicle Dispersions. The number of hydrogen ions bound to vesicle bilayers in the presence of KCl as a function of pH was estimated from titration data obtained in the following manner. About 500 mg of lyophilized phosphatidylcholine was suspended in 12 ml of deionized water and sonicated as described above. After centrifugation to remove undispersed phospholipid, the supernatant was diluted with deionized water to a final concentration of 10 μmol of phospholipid/ml and used immediately in the titration experiments; 5-ml aliquots of freshly prepared vesicle solution were added into 5 ml of pure water, 5 ml of 1.0 *M* KCl, or 5 ml of 1.0 *M* NaF and titrated with either H_2SO_4 or KOH. Titrations were carried out over the pH range 2–11 with vigorous stirring at 20° using a Radiometer pH meter (Model 225) coupled to a Beckman chart recorder (Model 1005). All solutions were flushed with purified argon and the titration itself was carried out in an argon atmosphere to eliminate CO_2 and to minimize oxidation of the phospholipid. Under these conditions, the control titration data for deionized solvent water fell on the ideal line in a plot of pH vs. log of the added acid or base (Figure 2). This was not the case unless all solutions were flushed with argon and the water used as solvent was prepared by passage over both cation and anion exchange columns immediately prior to use. Analytical grade KCl (Fisher Scientific Company) and ultra pure NaF (E. Merck AG) were used as delivered.

Results and Discussion

Isotopic Chloride Flux. Typical ^{36}Cl flux data obtained at 6.3° and several pH values are shown in Figure 3. In this figure $(\Delta N/C_0^*)/(P_i)$ is plotted vs. *t*, where ΔN is the total

Table I: ^{36}Cl Permeability Coefficients.

Temp (°C)	Bilayer Membrane System: 1. Vesicle Dispersion Egg Phosphatidylcholine[a] k_{Cl} (cm/sec)	2. Spherical Bilayer Diphytanoylphosphatidylcholine[b] k_{Cl} (cm/sec)	3. Spherical Bilayer Egg Phosphatidylcholine in Mixed Solvent[c] k_{Cl} (cm/sec)
3.2	1.5×10^{-11}		
6.3	3.1×10^{-11}		
10.0	2.6×10^{-11}		1.3×10^{-7}
11.0	4.5×10^{-11}		
15.0	6.1×10^{-11}		
20.0	11.3×10^{-11}	6.8×10^{-8}	2.4×10^{-7}
30.0			4.5×10^{-7}

[a] Aqueous phase unbuffered 2.0 *M* KCl (pH 5.5—6.0). [b] Aqueous phase unbuffered 0.1 *M* KCl (pH 5.5—6.0) (Toyoshima and Thompson, 1975). [c] Aqueous phase unbuffered 0.2 *M* NaCl (pH 5.5—6.0) (Pagano and Thompson, 1968).

^{36}Cl in cpm which crossed the vesicle bilayers in time t, C_0^* is the concentration of ^{36}Cl in cpm/ml in the external aqueous phase, and (P_i) is the concentration of phospholipid in moles of phosphorus/ml. At each pH the data fit a simple saturation curve with a plateau region corresponding to the equilibrium condition of equal ^{36}Cl concentrations on both sides of the bilayer. Under the assumption that ^{36}Cl permeation follows first-order kinetics, the permeability coefficient, k_{Cl}, was calculated from

$$-\ln(1 - \Delta N/\Delta N_\infty) = (A/V)k_{Cl}t \qquad (1)$$

Here ΔN and ΔN_∞ are concentrations of ^{36}Cl, in cpm/ml, contained in the vesicles at time t and at equilibrium, respectively. A is the surface area and V the internal volume of the vesicle.

In order to calculate the permeability coefficient k_{Cl}, the vesicle volume and surface area are required. Estimates of these parameters were obtained as follows: Johnson and coworkers (1971) calculated the surface area per phosphatidylcholine molecule to be 66 ± 3 Å^2 from surface potential measurements. A value of 71.7 Å^2/molecule was determined by Small (1967) from X-ray diffraction data. The surface area per phosphatidylcholine molecule in a collapsed monolayer was found by Shah and Schulman (1965) to be 62 Å^2. A value of $(1.9 \pm 0.2) \times 10^3$ cm^2/mol of P_i can be calculated from the average of these three values. The internal aqueous volume of the vesicle system required for the calculation of k_{Cl} was found to be $(1.6 \pm 0.1) \times 10^{-4}$ ml/mol of P_i from the plateau values in Figure 3. This value is essentially equal to the internal vesicle volume of 1.67×10^{-4} ml/mol of P_i obtained for similar preparations in our laboratory based on the amount of glucose trapped during preparative sonication (Dawidowicz, 1974). It is also in very good agreement with a value of 1.6×10^{-4} ml/mol of P_i determined by Kornberg and coworkers (1972) from spin-label data. Recently Hauser et al. (1972) have reported a value of $3.3 \pm 0.1 \times 10^{-4}$ ml/mol of P_i for the internal vesicle volume determined from the amount of trapped ^{36}Cl. These investigators, however, used an unfractionated dispersion. Their larger value for the internal volume is probably due to the presence of large vesicles in their preparation which frequently have average internal volumes as large as 12×10^{-4} ml/mol of P_i (Huang, 1969; Dawidowicz, 1974). All chloride permeability coefficients were calculated using a value of $V/A = (0.84 \pm 0.3) \times 10^{-7}$ cm based on a surface area of $(1.9 \pm 0.1) \times 10^3$ cm^2/mol of P_i and an internal volume of $(1.6 \pm 0.1) \times 10^{-4}$ ml/mol of P_i.

Temperature Dependence of ^{36}Cl Permeability. ^{36}Cl permeability coefficients are listed as a function of temperature in Table I, column 1. The relative error to be associated with these values is ±30%. Within this limit of error the value of k_{Cl} of 1.5×10^{-11} cm/sec at 3.2° is in reasonable agreement with the values obtained at 4° by Hauser and coworkers (1972, 1973) for ^{36}Cl efflux in unfractionated liposomes in 0.145 *M* NaCl, over the pH range 3.1–10.0. For comparison, Table I lists in column 2 the values of k_{Cl} obtained for semispherical bilayers (Toyoshima and Thompson, 1975), and in column 3 values obtained for spherical bilayers (Pagano and Thompson, 1968). It is apparent that k_{Cl} is very much larger in bilayers containing an organic solvent than it is in the solvent-free vesicle bilayer.

An Arrhenius plot of the data listed in Table I, column 1, is linear over the temperature range 3–20°. The activation energy calculated from the slope of the least-squares line is 19 ± 2 kcal/mol. The corresponding value calculated from the data listed in Table I, column 3, for spherical bilayers formed from egg phosphatidylcholine, *n*-tetradecane, methanol, and chloroform is 10.7 ± 0.4 kcal/mol (Pagano and Thompson, 1968). This substantially smaller activation energy may also be due to the presence of organic solvents in these bilayers. A very much smaller activation energy of 4.0 kcal/mol has been reported by Papahadjopoulos and Wadkins (1967) for chloride permeability in multilamellar liposomes formed from egg phosphatidylcholine. Although the reason for the low value is unknown, it may be due to the presence of impurities in the phosphatidylcholine or to gross leaks in the multilamellar structures.

Exchange and Net Fluxes of ^{36}Cl. In the preceding paper (Toyoshima and Thompson, 1975) and in earlier studies (Pagano and Thompson, 1968) it was shown in semispherical and spherical bilayer systems that the influx of ^{36}Cl was markedly reduced if NO_3^- was substituted for Cl^- on the trans side of the bilayer (inside aqueous compartment), but was unaffected by a cis substitution of NO_3^- for Cl^-. Figure 4 presents the results of analogous experiments carried out on bilayer vesicles. It is apparent that these results are qualitatively similar to those obtained with the other two bilayer systems. The effects of trans and cis substitution of NO_3^- or Cl^- on the ^{36}Cl flux in all three systems provide strong evidence for the coupling of inward and outward chloride fluxes. Additional evidence for coupling is provided by the data designated by circles in Figure 5. Plotted in this figure are the ratios of the concentration ^{36}Cl inside the vesicle to that in the external aqueous phase

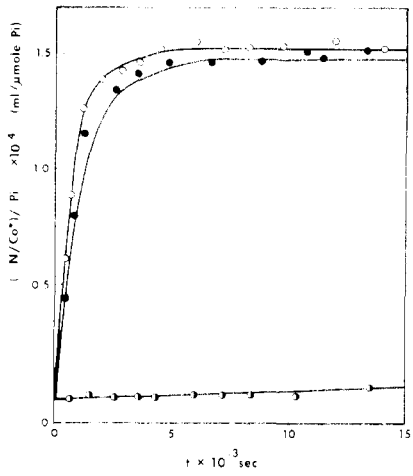

FIGURE 4: The effect of nitrate substitution for chloride on the ^{36}Cl influx. (●) External aqueous solution 1.0 *M* KCl, internal aqueous solution 1.0 *M* KCl; (○) external aqueous solution 1.0 *M* KNO_3, internal aqueous solution 1.0 *M* KCl; (◑) external aqueous solution 1.0 *M* KCl, internal aqueous solution 1.0 *M* KNO_3.

(C_i*/C_o*) as a function of time for three unbuffered concentrations of cold chloride. It is apparent that, on the time scale of these experiments, plateau values for the ratio C_i*/C_o* are not unity for total cold chloride concentrations of 0.01 *M* KCl (◑) and 0.001 *M* KCl (○), but approach 1 for 1.0 *M* KCl (●). For these data and those obtained for similar experiments, the plateau values for the isotope ratio, $C_{i\infty}$*/$C_{o\infty}$*, are fitted by the following equation:

$$C_{i\infty}^*/C_{o\infty}^* = C_{Cl,0}/\{C_{Cl,0} + C_{o,0}^*\} \quad (2)$$

Here $C_{Cl,0}$ is the initial concentration of cold chloride before addition of ^{36}Cl and $C_{o,0}$* is the initial concentration of chloride added with the ^{36}Cl. Thus, the right-hand side of eq 2 is the ratio of the concentration of total chloride inside to the concentration of total chloride outside the vesicle initially. Because of the addition of the ^{36}Cl and its carrier chloride, the denominator is larger than the numerator in eq 2. The ratio is in fact a measure of the chemical chloride gradient which exists across the vesicle bilayer at zero time. These results clearly show that in the absence of buffer, ^{36}Cl permeation is the result of the exchange of external ^{36}Cl for internal cold chloride and that the permeation of ^{36}Cl caused by the net flux of chloride down the chemical gradient is too slow to be detected on the time scale of these experiments.

The effect of buffer on the ^{36}Cl flux is shown by the data designated by triangles in Figure 5. Under the conditions of the experiment (0.1 *M* KOAc buffer, no chloride, pH 6.0) a net flux of chloride occurs uninterruptedly over 75 × 10^3 sec until the total chloride concentration gradient disappears and $C_{i\infty}$*/$C_{o\infty}$* = 1.

The results presented in Figure 5 together with the effects of NO_3^- on ^{36}Cl permeation shown in Figure 4 can be explained if it is assumed that the net chloride flux is coupled to a proton flux. Thus, in the absence of a buffer, this net coupled flux of H^+ and Cl^- generated by the transbilayer chloride concentration difference gives rise to a transbilayer pH gradient which quickly reduces the net H^+Cl^- flux to zero. However, in the presence of buffer, no pH gradient can form and hence the slow net flux of HCl continues until the chloride gradient is dissipated (triangles in Figure 5).

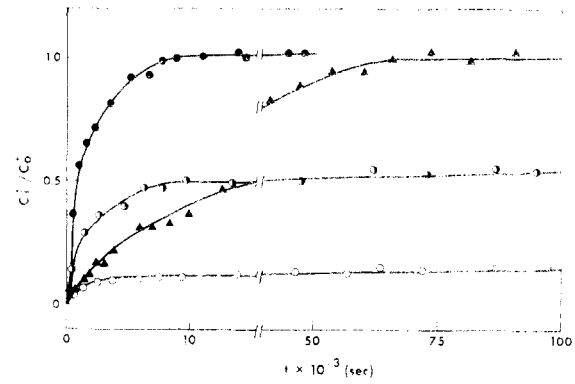

FIGURE 5: The effects of cold chloride concentration and buffer on ^{36}Cl permeation at 6.3°. (●) Aqueous phase 1.0 *M* KCl (no buffer), (◑) aqueous phase 0.01 *M* KCl (no pH buffer), (○) aqueous phases 0.001 *M* KCl (no buffer), (△) 0.1 *M* potassium citrate buffer, pH 6.0 (no cold Cl^-).

If the net flux of chloride is coupled to a flux of protons, then for the unbuffered experiments presented in Figure 5 (data depicted by circles) the distribution of Cl^- and H^+ in the interior and exterior aqueous phases at equilibrium is given by

$$C_{H^+,0,\infty}/C_{H^+,i,\infty} = \{C_{Cl,i,\infty} + C_{i,\infty}^*\}/\{C_{Cl,0,\infty} + C_{o,\infty}^*\} = C_{i,\infty}^*/C_{o,\infty}^* \quad (3)$$

Here $C_{H^+,0,\infty}$ and $C_{H^+,i,\infty}$ are the concentrations at equilibrium of H^+ in the inner and outer aqueous compartments, respectively, and $C_{Cl,0,\infty}$ and $C_{Cl,i,\infty}$ are the corresponding values for cold chloride. This equation does not explicitly involve the concentrations of K^+ in the internal aqueous compartment since the permeability of the vesicle bilayer is much smaller for K^+ than for HCl (Toyoshima and Thompson, 1975). In addition, the second equality in eq 3 is based on the observation that the exchange permeation of ^{36}Cl is much faster than the net permeation of HCl. This point will be discussed more fully later. If a 1:1 stoichiometry of Cl^- and H^+ movements is assumed, then eq 3 leads to eq 2 as a final expression for $C_{i,\infty}$*/$C_{o,\infty}$*. Thus, it seems reasonable that the net flux of chloride is in fact coupled to a hydrogen flux with 1:1 stoichiometry. It is interesting to note that, as the result of an analysis of the effects of pH and chloride concentration on the tempotartrate distribution between the inside and outside of egg phosphatidylcholine vesicles, Kornberg and coworkers (1972) found a 1:1 stoichiometry of Cl^- and H^+ movements in the net flux of H^+ across the bilayer.

Coupling of the net chloride flux to a stoichiometric flux of protons could in principle be electrical, that is determined by the electroneutrality condition, or be the result of molecular HCl, or HCl–carrier complex permeation. If coupling were electrical, then the permeability of H^+ should be equal to that of chloride. Unfortunately the permeability of the bilayer to H^+ cannot be determined experimentally. However, it is reasonable to expect that, in the absence of a special mechanism, the permeabilities of the bilayer to H^+ and Na^+ should be approximately equal. Since the experimentally determined values of k_{Na^+} and k_{Cl^-} have been shown to differ by more than two orders of magnitude in spherical bilayers (Pagano and Thompson, 1968) and vesicles (Hauser et al., 1973), coupling by ion pairing (Cl^- and H^+) seems very unlikely. This conclusion is supported by the observations that the isotopic chloride

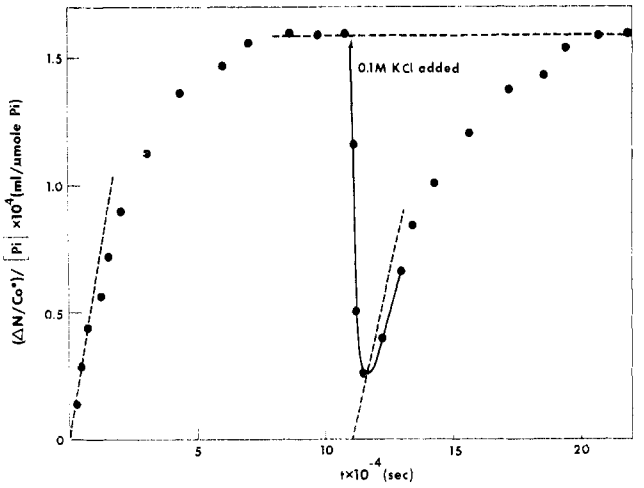

FIGURE 6: The effect of added external cold Cl^- on the influx of ^{36}Cl. 0.1 *M* KCl (unlabeled chloride) was added at $t = 11 \times 10^4$ sec; 0.5 *M* potassium maleate buffer (pH 6.0) (see text for details).

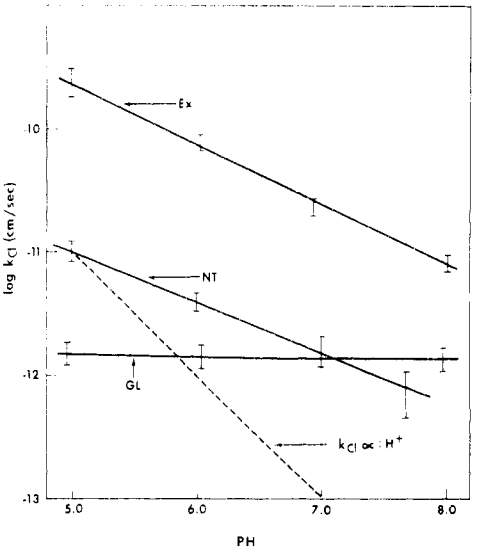

FIGURE 7: pH dependence of chloride permeability. EX, exchange diffusion obtained in 1.0 *M* KCl and 0.1 *M* buffer solutions at 6.3°; NT, permeability of the net chloride flux measured in 0.1 *M* buffer solution in the absence of cold chloride at 6.3°; GL, glucose permeability of the vesicle bilayer obtained in 1.0 *M* KCl and 0.1 *M* pH buffer solutions at 6.3°. Aqueous phase compositions same as Figure 3.

flux through spherical and semispherical bilayers is electrically silent (Pagano and Thompson, 1968; Toyoshima and Thompson, 1975). Thus it seems that the net chloride flux and its coupled hydrogen flux must be the result of either molecular HCl and HCl–carrier permeation.

Examination of the data in Figure 5 shows that the rate constant for net chloride influx coupled to proton permeation is smaller by a factor of about 20 than the rate constant for ^{36}Cl exchange. This marked difference in rates for the two permeation processes is clearly demonstrated in a different type of experiment. The results of this experiment are presented in Figure 6. Vesicles were initially prepared in 0.5 *M* potassium maleate buffer at pH 6 in the absence of chloride. After addition of ^{36}Cl to the external phase at time zero to give a final concentration of 10^{-2} *M*, the slow net permeation of H and ^{36}Cl occurred as shown by the saturation curve on the left-hand side of Figure 6. The ordinate value of 1.58×10^{-4} ml/mol of P_i in the plateau region lying between (6 and 10) $\times 10^4$ sec indicates that equilibration of ^{36}Cl between interior and exterior aqueous phases has occurred (cf. Figure 3). At 11×10^4 sec (arrow) cold KCl was added to the external aqueous phase to give a final concentration of 0.1 *M*. The rapid decrease of ^{36}Cl in the internal vesicle compartment must be the result of the exchange of ^{36}Cl contained within the vesicles with the added cold chloride in the external aqueous phase. The subsubsequent slow recovery of internal ^{36}Cl is due to net permeation of H and ^{36} Cl paralleling the net inward flux of H and Cl driven by the transbilayer concentration gradient established at 11×10^4 sec.

These data clearly show that the rate constant for the ^{36}Cl exchange flux is at least one order of magnitude greater than that for the coupled net (H and Cl) fluxes. The results also provide strong evidence against the proposition that the exchange chloride flux is due to molecular HCl permeation. If the chloride exchange flux were simply composed of two opposing fluxes of molecular HCl, then no abrupt change of ^{36}Cl concentration on the inside of the vesicle would occur when cold chloride was added to the external solution. This would be the case since the pH on both sides of the vesicle bilayer was maintained constant by the buffer and hence the outward and inward fluxes of $H^{36}Cl$ would be independent of the concentration of cold chloride. This is clearly the result shown in Figure 6. It is important to note that the buffer concentration of 0.5 *M* was substantially larger than the 0.1 *M* concentration of added cold chloride.

Effect of pH on ^{36}Cl Net and Exchange Fluxes. Additional insight into the mechanism of the chloride exchange and net fluxes was obtained from an examination of the dependence of these fluxes on pH. The exchange flux was determined at several pH values in buffered 1.0 *M* KCl solutions. The net chloride flux was also determined at these pH values in similar buffer systems but in the absence of cold chloride (0.1 *M* buffer at pH 5, potassium acetate; pH 6, potassium citrate; pH 7 and 8, Tris nitrate). The results of these studies are shown in Figure 7 in which log of the chloride permeability coefficient k_{Cl} is plotted vs. pH. The data for both fluxes are best fitted by straight lines with equal negative slopes. However, the magnitude of k_{Cl} for the exchange flux is 20-fold greater than the corresponding value for the net flux over the pH range 5–8. Also shown in Figure 7 is the glucose permeability of identical vesicles examined under similar conditions. The fact that glucose permeability does not depend on pH strongly suggests that the permeability properties of the vesicle bilayer per se do not change with pH. The data for the exchange flux shown in Figure 9 are in general agreement with the less extensive results reported by Hauser and coworkers (1973) obtained under somewhat different conditions.

Perhaps the most interesting result shown in Figure 7 is the fact that the slopes of the curves for both the exchange and net chloride fluxes are 0.5. Recently Kornberg and coworkers (1972) have reported that the rate of equilibration of tempotartrate across bilayers of egg phosphatidylcholine vesicles in the presence of Cl^- or valinomycin-K^+ was proportional to pH. From these results, they concluded that tempotartrate permeated the vesicle bilayer in its uncharged acidic form. If the mechanism of chloride permeation were due to fluxes of molecular HCl, then by analogy to tempotartrate permeation, the slopes of the log k_{Cl} vs. pH plots would be expected to be unity. The value of 0.5 obtained for the slopes of these plots argues against a molecu-

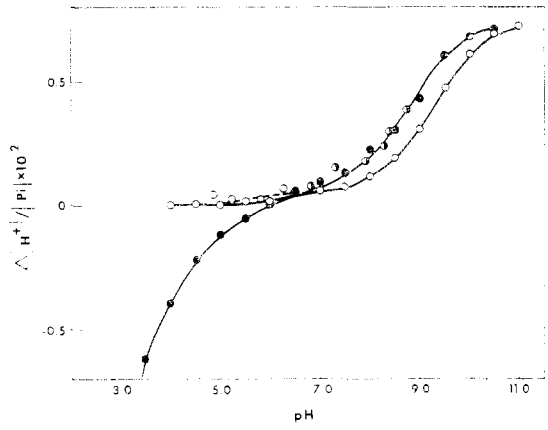

FIGURE 8: Titration data for the vesicle dispersions. (○) Pure water; (◑) 0.5 *M* NaF; (●) 0.5 *M* KCl at 21°.

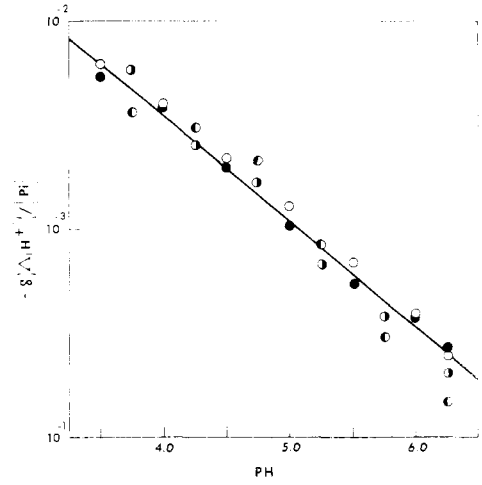

FIGURE 9: The pH dependence of chloride-induced proton binding to phosphatidylcholine vesicles derived from the data in Figure 10. The different symbols denote data obtained in separate experiments.

lar HCl permeation mechanism. However, as discussed above the net flux of chloride was clearly demonstrated to be coupled to a proton flux with 1:1 stoichiometry. The identical pH dependence of the exchange and the net chloride fluxes suggests that the exchange flux as well as the net flux is coupled to a proton flux with 1:1 stoichiometry.

Chloride-Dependent Proton Binding to Vesicles. Since the flux of ^{36}Cl through the vesicle bilayer would be expected to be proportional to the concentration of chloride at the bilayer surface, the data presented in the preceding section suggest that interdependent chloride and proton binding occurs at the bilayer surface. The results of titrations carried out in vesicle dispersions both in the presence and absence of chloride do in fact show that proton binding by the vesicle bilayer is induced by Cl^- added to the external aqueous phase.

Representative titration curves for pure water (filled circles), egg phosphatidylcholine vesicles in pure water (open circles), and vesicles in 0.5 *M* KCl (half-filled circles) are shown in Figure 2. These data, together with additional data for a vesicle dispersion in 0.5 *M* NaF, are used to generate the curves presented in Figure 8. In this figure the moles of protons dissociated per mole of vesicle phosphatidylcholine are plotted vs. the pH of the external aqueous phase. The data for the salt-free vesicle dispersion (open circles) clearly show that the vesicles contain some titratable material with a p*K* of about 9. Since phosphatidylcholine has no titratable groups in this pH range, the uptake of protons is probably due to the presence of oxidation products formed during sonication. That this is in fact the case is supported by the observation that the amount of titratable material relative to total phospholipid increases somewhat with the time of sonication. In addition, deliberate oxidation of vesicle dispersions was found to greatly increase the amount of titratable material.

The presence of 0.5 *M* NaF in aqueous phases (half-filled circles, Figure 8) shifts the vesicle titration curve to lower pH values by about 0.5 unit, but does not alter its shape. The titration data for vesicle dispersions containing 0.5 *M* KCl (solid circles), however, show an additional titration in pH range of 3.0–7.0. The data in the pH range 7.0–10.0 within experimental error fall on the curve obtained in 0.5 *M* NaF. While it seems probable that the shift of the titration curve in the pH range 7–10 is an ionic strength effect on the p*K* of the oxidation products, proton uptake in the presence of chloride in the low pH range 3.0–7.0 appears to be specifically induced by chloride.

The magnitude of the chloride-dependent proton binding as a function of pH is shown in Figure 9. The ordinate in this figure, the log number of protons bound per mole of lipid phosphorus, was calculated from the differences between the titration curve in 0.5 *M* KCl and the titration curve for the vesicles in pure water corrected for the small, but nonzero, contribution of the oxidation products (dashed line, Figure 8). It is quite apparent that data in Figure 9 are well fitted by a straight line over the pH range 3–6.5. In this figure, the different symbols denote data obtained in separate experiments. Unfortunately the precision of the primary data shown in Figure 8 made it impossible to calculate a meaningful difference curve above pH 6.5.

The most important result obtained from the data in Figure 9 is the fact that the slope of the least-squares line is −0.5. Thus the pH dependence of the chloride-dependent proton binding is the same as the pH dependence of both the exchange and net chloride fluxes. This correspondence of the pH dependence of chloride-induced proton binding and chloride permeation strongly suggests that the H^+ and Cl^- interdependently bound to the phosphatidylcholine of the vesicle bilayer are the identical ions involved in chloride permeation. Stated differently, interdependent H^+ and Cl^- binding by phosphatidylcholine must precede transbilayer movement of these species.

The Mechanism of Chloride Permeation. Pagano and Thompson (1968) first suggested that a chloride–phospholipid complex is the transport species giving rise to the large electrically silent ^{36}Cl flux in phospholipid bilayer systems. Based on measurement of transbilayer "flip-flop" of phosphatidylcholine in vesicle systems determined by electron spin resonance techniques, Kornberg and McConnell (1971) have recently suggested that this flux may be associated with "flip-flop". The results reported above are entirely consistent with these suggestions, provided that the transport or "flip-flop" species is a phospholipid–HCl complex.

Although a quantitative formulation of the molecular mechanism of exchange permeation is not yet possible, the process may be described qualitatively as follows. In chloride solution a small fraction of the phosphatidycholine molecules in the vesicle bilayer are each associated with a tightly bound chloride ion. The bound chloride (presumably on the quaternary ammonium group) induces proton binding (presumably on the phosphate group) of the same mole-

cule, forming in effect the hydrochloride of phosphatidylcholine. It is this phosphatidylcholine-bound H^+Cl^- which is the actual species involved in chloride permeation. If this mechanism is correct, the flux of chloride in the form of the phospholipid–HCl complex should be proportional to the chloride-induced proton binding to phospholipid which has been shown to be proportional to $[H^+]^{1/2}$. The data presented above show that this is in fact the case. The mechanism is not only consistent with all the data presented in this paper, but it is also entirely consistent with the available data for the electrically silent ^{36}Cl flux obtained in semispherical bilayers (Toyoshima and Thompson, 1975) and in spherical bilayer systems (Pagano and Thompson, 1968). In addition, recent proton nuclear magnetic resonance studies have provided independent evidence of Cl^- binding to the quaternary ammonium group of phosphatidylcholine (Jendrasiak, 1972).

It is of interest to make a quantitative comparison of the ^{36}Cl permeability parameters with the corresponding parameters derived from the "flip-flop" data of Kornberg and McConnell (1971) obtained with similar vesicles. These authors have reported a "flip-flop" activation energy of 19.4 kcal/mol at 30° in an aqueous phase containing 0.05 *M* Tris and 0.1 *M* NaCl (pH 8.0). The activation energy for ^{36}Cl permeation obtained in 2 *M* KCl at pH 5.5–6.0 in the temperature range 3–20° is 19 ±2 kcal/mol. The correspondence of these activation energies suggests that the principal molecular species engaged in transbilayer "flip-flop" may be the phosphatidylcholine–HCl complex. If this is assumed to be the case then the magnitude of the chloride flux associated with "flip-flop" can be estimated as follows. The largest value of the rate constant for phospholipid "flip-flop" motion in the egg phosphatidycholine vesicles reported by Kornberg and McConnell (1971) is 0.403 hr^{-1}. This value obtained at 30° in 0.1 *M* NaCl (pH 8.0) corresponds to an unidirectional translocation of 1.7×10^{-14} mol of phospholipid per cm^2 per sec and hence to a chloride flux of the same value. The permeability constant for the chloride exchange flux at 6.3° in 1.0 *M* KCl–0.1 *M* Tris (pH 8.0) is 7.9×10^{-12} cm/sec (Figure 9). If it is assumed that this permeability coefficient is independent of the concentration of Cl^- in the ambient aqueous phase, then a chloride exchange flux of 0.79×10^{-16} mol per cm^2 per sec can be calculated for 0.1 *M* KCl (pH 8.0), 6.3°. If in addition the reasonable assumption is made that chloride permeation has an activation energy of 19 ± 2 kcal/ml under these conditions, a corresponding unidirectional chloride exchange flux at 30° is calculated to be 1.5×10^{-14} mol per cm^2 per sec. The close agreement between this value and the value of 1.7×10^{-14} mol per cm^2 per sec estimated from the "flip-flop" parameters is consistent with the suggestion that essentially all of the ^{36}Cl flux is associated with transbilayer "flip-flop". If this is in fact the case then phosphatidylcholine "flip-flop" should be dependent on both the chloride concentration and pH of the ambient aqueous phase. NMR studies utilizing paramagnetic shift reagents and vesicles formed with a transbilayer compositional asymmetry are currently in progress in order to assess the dependence of "flip-flop" on $[Cl^-]$ and pH.

A plausible mechanism for the ^{36}Cl net flux in cold chloride-free systems is more difficult to formulate. While the data presented above rule out the possibility that the exchange flux of ^{36}Cl depends on the permeation of molecular HCl, such is not the case with the net ^{36}Cl flux. The pH dependence, however, argues that even the net flux also involves a phosphatidylcholine–HCl complex as a major permanent species.

An electrically silent chloride transport system has recently been shown to be present in the membranes of mammalian erythrocytes (Tosteson, 1959; Gunn et al., 1973). The permeability to chloride conferred on the membranes by this system is four to six orders of magnitude larger than the chloride ion permeability estimated from electrical parameters. In this respect the erythrocyte membrane differs from frog sartorius muscle and squid axon which have k_{Cl} values of 1.2×10^{-7} cm/sec (Sperelakis, 1969) and 1.0×10^{-8} cm/sec (Jain, 1972), respectively. Although the chloride permeability of the erythrocyte membrane is qualitatively similar to that displayed by phosphatidylcholine vesicles and spherical bilayers, there is abundant evidence that the erythrocyte transport system is comprised of polypeptide components. In addition, since the magnitude of the chloride permeability coefficient in the red cell is at least 10^2 larger than k_{Cl} determined in bilayer systems (Jain, 1972), it seems unlikely that transport based on a phosphatidylcholine–HCl complex can contribute measurably to erythrocyte chloride transport. Such may not, however, be the case for other biological membranes such as squid axon and muscle that have chloride permeabilities comparable to the bilayer systems.

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