as a minor ganglioside of brain (Svennerholm, 1963) while AG_5, the major NANGly counterpart, has not yet been detected in brain. Additional minor gangliosides are present in the adrenal medulla, as indicated by unidentified TLC bands and the presence of a small amount of glucosamine detected in the hexosamine fraction. The five species elucidated here are estimated to comprise well over 95% of total ganglioside in this tissue. While this study has pointed up many similarities to brain gangliosides the overall pattern, particularly in regard to hematoside predominance, sialic acid type and lipophilic composition (Ledeen and Salsman, 1970), indicate a closer relationship to gangliosides of extraneural organs (for review, see Ledeen and Yu, 1973).

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Chloride Flux in Bilayer Membranes: The Electrically Silent Chloride Flux in Semispherical Bilayers†

Yoshinori Toyoshima‡ and T. E. Thompson*

ABSTRACT: High resistance semispherical bilayer membranes of areas as large as 0.3 cm^2 were formed from a decane solution of synthetic diphytanoylphosphatidylcholine. These bilayers had a specific resistance of about 10^9 Ω cm^2 and a specific capacitance of 0.38 μF cm^{-2} at 20° in 0.1 *M* KCl. Under these conditions, chloride permeability was 6.8×10^{-8} cm/sec. This electrically silent ^{36}Cl flux was found to be about 10^3-fold larger than the chloride current calculated from the electrical parameters of the system. The chloride flux in the bilayer was independent of the applied electrical field and was unaltered by addition of reducing agents to the ambient aqueous solutions. It was, however, substantially reduced when NO_3^- was substituted for Cl^- on the side of the bilayer initially free of ^{36}Cl, or if I^- was added to the aqueous phases in the concentration range of 0.001-0.1 *M*. These results strongly suggest that the electrically silent flux of ^{36}Cl is primarily a carrier mediated diffusion process in which phosphatidylcholine acts as the carrier species.

Most current concepts of biological membrane structure are based on the premise that the phospholipid component is present in bilayer form and as such constitutes a barrier matrix for the organization of protein and carbohydrate components. Because of this central position of the bilayer in membrane structure, extensive studies of the molecular organization and physical properties of bilayers have been carried out in many laboratories (Tien and Diana, 1968; Rothfield and Finkelstein, 1968; Mueller and Rudin, 1969; Thompson and Henn, 1970; Bangham, 1972). Considerable attention has been directed toward the permeabilities of bilayers to cations, but relatively little attention has been given to anion permeabilities.

Pagano and Thompson studied chloride permeability in spherical bilayers of large area by both electrical and isoto-

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pic techniques. Those bilayers which had relatively low specific resistances of about 10^6 Ω cm^2 at 30° displayed an electrically silent ^{36}Cl flux about 10^2-fold larger than the chloride current calculated from the electrical parameters of the system (Pagano and Thompson, 1967, 1968). Although this flux appeared to be carrier mediated, the complex composition of the bilayers, which were formed from egg phosphatidylcholine and *n*-tetradecane in a chloroform–methanol solvent, made identification of the carrier uncertain. In addition, it proved impossible to carry out electrical and isotopic measurements simultaneously on the same bilayer and thus establish the electric field dependence of the ^{36}Cl flux. Repeated attempts to form spherical bilayers of this type from a solution of a pure phospholipid in decane were uniformly unsuccessful. Apparently in the Pagano–Thompson system the density difference between the membrane-forming solution containing chloroform and the ambient aqueous phases was so small that buoyancy forces did not cause bilayer rupture during thinning. However, with membrane-forming solutions of simple composition such was not the case. The minimum attainable density difference with phospholipid–decane solutions generated buoyancy forces large enough to invariably cause bilayer rupture.

We report here chloride permeability studies on mechanically stable semispherical bilayers of large area formed from synthetic diphytanoylphosphatidylcholine and *n*-decane. The design of this bilayer system is such that even large buoyancy forces do not lead to bilayer breakage, and, in addition, isotopic flux and electrical parameters can be determined simultaneously on individual bilayers. These high resistance bilayer membranes of simple composition also show an electrically silent ^{36}Cl flux. The available evidence strongly suggests that this flux is carrier mediated and that phosphatidylcholine is the carrier molecule (Toyoshima and Thompson, 1972).

Experimental Section

Bilayer Formation. Bilayers were formed from chromatographically pure synthetic 1,2-diphytanoyl-3-*sn*-phosphatidylcholine in olefin-free *n*-decane. Preparation of the phosphatidylcholine has been described elsewhere (Redwood et al., 1971). The *n*-decane obtained from Lachat Chemicals, Inc. (purity 99.5%) was routinely chromatographed on activated aluminum prior to use.

Semispherical bilayers were generated by a modification of the technique first described by Tsofina and coworkers (Tsofina et al., 1966). The basic design of the system is illustrated in Figure 1. The membrane-forming solution of phosphatidylcholine in *n*-decane was floated on a small volume of aqueous solution. A short length of Teflon tubing, 1.5 mm i.d., was positioned so that its top was in the lipid phase. The other end of the Teflon tubing was sealed to a short length of glass tubing which, in turn, was connected to a microsyringe. This tubing and syringe system were filled with the appropriate aqueous solution. The internal aqueous phase, was, however, subdivided into two compartments (R and T, Figure 1) by a small cap of lipid phase in the Teflon tubing positioned about 5 mm from the lower tip. To make a bilayer membrane, the aqueous solution in the Teflon tubing was very slowly extruded into the lipid phase to form a shell-film just below the interface between the lipid phase and the aqueous solution on which it floated. Within 5 min, the shell-film usually began to thin to bilayer thickness at the bottom of the semisphere. The growing bilayer area gradually spread to occupy the entire area of the semisphere as the excess lipid in the shell-film was extruded into the bulk lipid layer. This process was usually complete within 10 min. The entire system shown schematically in Figure 1 was thermostatically controlled at 20 ± 0.5°. In all experiments, the bilayer was observed through a 40 power microscope with calibrated reticle. From measurements of the chord, *d*, of the membrane semisphere and its height, *h* (Figure 1), the area, *A*, of the bilayer segment was calculated from the formula $A = \pi\{(d/2)^2 + h^2\}$. In the studies described in this paper, membrane areas ranged from 0.02 to 0.3 cm^2.

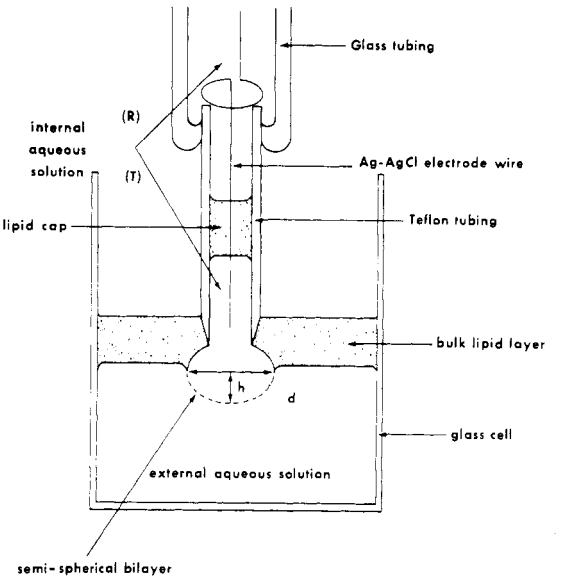

FIGURE 1: Vertical cross section of a semispherical bilayer. Chord of semispherical bilayer, *d*, and height *h*; internal aqueous phases, R and T; stirring bar, S.

Electrical Measurements. A block diagram of the apparatus used in studying the electrical properties of the spherical bilayer is shown in Figure 2. The two aqueous solutions, R and T, separated by the lipid cap in the Teflon tubing were maintained at equipotential by means of a fine silver–silver chloride wire, E_o, mounted in the Teflon tubing so as to pass completely through the lipid cap. Bilayer capacitance was monitored at 100 Hz using a Wayne–Kerr universal bridge (B221) coupled to a Tektronix oscilloscope (Type 502 A). The ac voltage across the bilayer, established by a pair of platinized-platinum electrodes, E_1 and E_4, was always less than 50 mV. Under these conditions, capacitance was independent of the applied voltage. A simple potentiometer connected to silver–silver chloride electrodes E_2 and E_5 was used to establish a transmembrane potential. Current in this circuit was measured with an E. H. Research Laboratories electrometer amplifier (Model 2010), coupled to a Beckman chart recorder (Model 1005). A second pair of silver–silver chloride electrodes, E_3 and E_6, connected to a Keithly Instruments high imput impedance electrometer (Type 610CR) were used to measure the transmembrane potential difference. Bilayer conductances were calculated from the initial slope of the current–voltage curve.

Diffusion potentials resulting from a transmembrane potassium chloride concentration gradient were also determined. The concentration of potassium chloride in the internal solution, C_i, was fixed at 0.1 *M* and that in the external solution, C_o, was varied to give the ratio C_o/C_i values of 1.25, 1.50, 1.75, and 2.0. Permeability coefficients for the

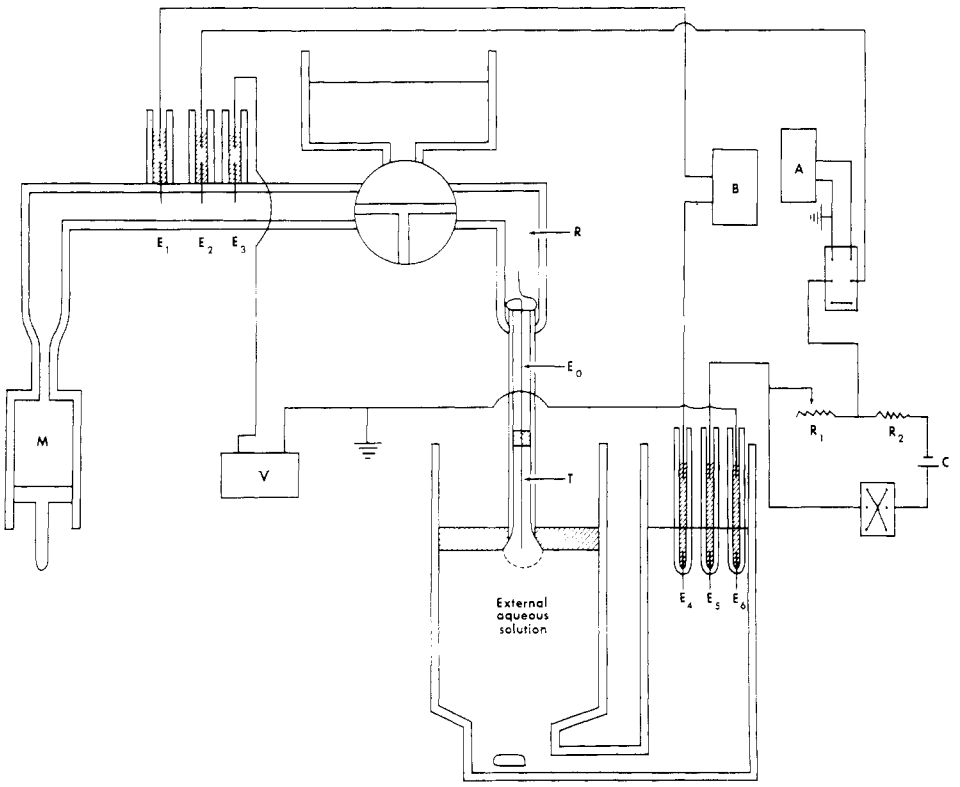

FIGURE 2: A block circuit diagram of the apparatus used to study the electrical parameters of the semispherical bilayer. Electrometer amplifier, A; Wayne-Kerr universal bridge, B; resistance, R_1 and R_2; E_0, E_{1-6}; Ag–AgCl electrodes; internal aqueous phases, R and T; high input impedance electrometer, V; microsyringe, M; stirring bar, S; mercury battery, C.

ionic species traversing the bilayer (k_+^{ion}, k_-^{ion}) were estimated from conductance and diffusion potential data using the following expressions (Kamo et al., 1971):

$$k_+^{ion} = (RT/2F^2)(G/C_i)\{1 - (F/RT)\Delta\phi_m/\ln(\gamma_o C_o/\gamma_i C_i)\} = (RT/F^2)(G/C_i)t_+$$

$$k_-^{ion} = (RT/2F^2)(G/C_i)\{1 + (F/RT)\Delta\phi_m/\ln(\gamma_o C_o/\gamma_i C_i)\} = (RT/F^2)(G/C_i)t_- \quad (1)$$

Here, the membrane potential $\Delta\phi_m = \Delta\phi_o - (RT/F)\ln(\gamma_i C_i/\gamma_o C_o)$ where $\Delta\phi_o$ is the observed potential difference between the two silver–silver chloride electrodes and γ_i and γ_o are the mean activity coefficients of potassium chloride in the internal and external solutions, respectively. F, R, and T are Faraday constant, gas constant, and absolute temperature, respectively. G is the specific conductance of the membrane (mho/cm^2), and t_+ and t_- are the transference numbers of cation and anion in the membrane system.

Isotopic Chloride Flux Measurements. In these experiments ^{36}Cl was always added to the external solution. The final concentration of the isotope was about 3 μCi/ml. After addition of ^{36}Cl a shell-film was formed as illustrated in Figure 3B. The spontaneous thinning of the film to a bilayer was followed both optically and by capacitance measurements. The capacitance was used to estimate bilayer area as a function of time in the thinning film and the area of the stable bilayer in its final configuration. Once the system reached its final configuration, the specific conductance of the membrane was determined. Usually values were in excess of 10^9 Ω cm^2. Occasional bilayers showed specific resistances 2–3 orders of magnitude lower than this value. Isotope flux measurements were carried out only on high resistance membranes.

After time Δt, the internal solution contained between the bilayer and the lipid cap in the Teflon tubing was sampled in the following way. The magnetic stirring bar (1 cm in length) set in the external solution was rotated very gently. The vortex formed by the rotating bar caused the bulk lipid solution to flow down the bilayer surface as shown in Figure 3C. Within 5 min, all bilayer area had become thick shell-film showing characteristic interference colors. At this point the solution contained in the shell was withdrawn into the Teflon tubing by means of the microsyringe so that the entire interior aqueous phase of the bilayer system was located within the Teflon tubing (Figure 3D). The Teflon tubing was then lifted out of the outer chamber by a rack and pinion. After thoroughly washing the outside of the Teflon tubing, the aqueous contents of the interior bilayer compartment were extruded onto a counting planchet. A Nuclear Chicago low background counter (Model 4334) was used in all experiments. The chloride permeability of the bilayer system was calculated from the data using the following equation:

$$k_{Cl} = J_{Cl}/C_o = J^*/C_o^* = (\Delta N/C_o^*)/\int_0^{\Delta t} A\,dt \quad (2)$$

where J_{Cl}, the inward flux of Cl in mol per cm^2 per sec, J^*, the inward flux of ^{36}Cl in cpm per cm^2 per sec, C_o, the concentration of Cl in mol/cm^3 in the external solution, C_o^*, the concentration of ^{36}Cl in cpm/cm^3 in the external solution, A, the area of the bilayer membrane in cm^2, ΔN, the total ^{36}Cl in cpm which crossed the membrane in time Δt, and k_{Cl}, the isotopic permeability of chloride in cm/sec.

Since the bilayer area expanded from zero to the final stable value during the initial part of the flux experiment, the integrated bilayer area $\int_0^{\Delta t} A\,dt$ was used in this equation. In all flux measurements, the concentration of ^{36}Cl in-

Table I: Electrical Properties of Three Types of Bilayers.

Properties	System 1. Diphytanoylphosphatidylcholine in *n*-Decane[a] Spherical Type	2. Diphytanoylphosphatidylcholine in *n*-Decane[b] Planar Type	3. Egg Phosphatidylcholine in *n*-Tetradecane, Chloroform, and Methanol[c] Spherical Type
C (μF/cm^2)	0.38 ± 0.02	0.40 ± 0.02	0.50 ± 0.03
G (mho/cm^2)	1.4×10^{-9}	$(1.1-9.1) \times 10^{-9}$	3.3×10^{-6}
t_+	0.59 ± 0.01		0.83 ± 0.01
t_-	0.41 ± 0.01		0.17 ± 0.01
k_+ion (cm/sec)	2.2×10^{-12}		7.5×10^{-9}
k_-ion (cm/sec)	1.6×10^{-12}		1.6×10^{-9}

[a] 0.1 *M* KCl at 20.0 ± 0.5°. [b] Data from Redwood et al. (1971): 0.1 *M* Tris-HCl (pH 7.5), 0.1 *M* NaCl, 0.1 *M* KCl, and 0.02 *M* $MgCl_2$ at 20–22°. [c] Data from Pagano and Thompson (1968): 0.2 *M* NaCl at 37°.

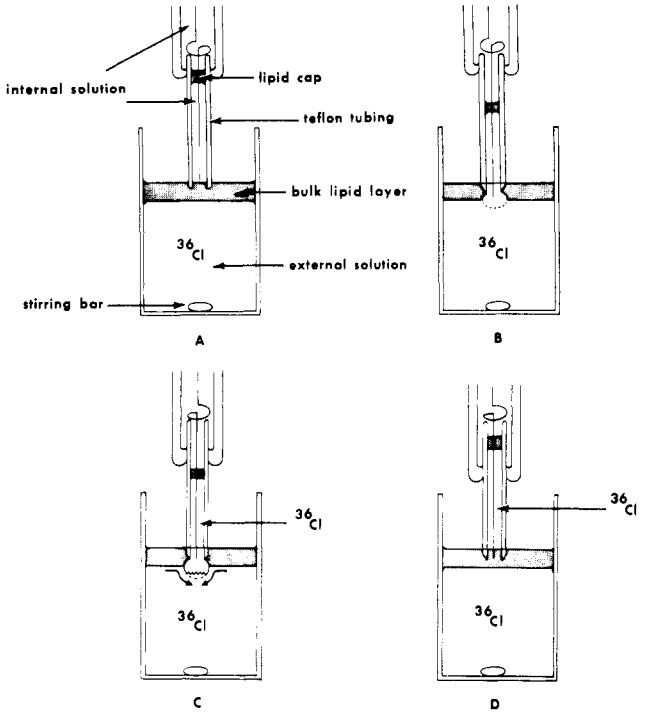

FIGURE 3: Experimental procedure for isotopic flux measurements. A, initial configuration of system before formation of the bilayer; B, configuration during flux experiment; C, re-formation of thick shell-film at the end of the flux experiment; D, withdrawal of internal aqueous phase into pipet tip. See text for details.

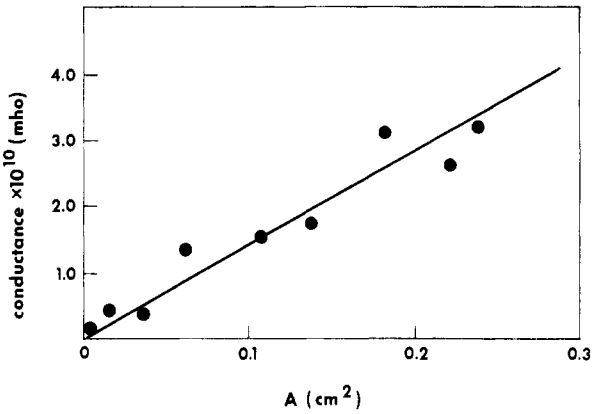

FIGURE 4: The measured system conductance at zero current vs. bilayer area. Each point represents a different bilayer. 0.1 *M* KCl, 20.0 ± 0.5°.

side the spherical shell at the end of the experiment was at least a 10^3-fold less than the concentration of ^{36}Cl in the external solution, thus back diffusion of ^{36}Cl was negligible. ^{36}Cl was obtained from New England Nuclear.

Results and Discussion

Electrical Properties. Table I, column 1, summarizes the electrical properties of semispherical bilayer membranes formed from diphytanoylphosphatidylcholine and *n*-decane. The current-voltage curve for this system showed a characteristic symmetrical sigmoidal shape identical with that reported for planar bilayers formed from the same synthetic phospholipid in *n*-decane (Redwood et al., 1971). Values of corresponding parameters for planar bilayers of similar composition (Redwood et al., 1971) and for low resistance spherical bilayers (Pagano and Thompson, 1968) are listed for comparison in columns 2 and 3, Table I. Figure 4 is a plot of membrane conductance determined from the initial slopes of current-voltage curves as a function of the bilayer area. Figure 4 shows the conductance to be a linear function of the bilayer area with the extrapolated conductance equal to zero at zero area. A value of 1.4×10^{-9} mho/cm^2 was calculated from these data for the specific conductance *G*. This value falls in the range reported by Redwood et al. (1971) for planar bilayers of the same composition (Table I, column 2). It is, however, three orders of magnitude smaller than the conductance obtained for egg phosphatidylcholine–tetradecane–chloroform–methanol bilayers (Table I, column 3). A plot of the membrane capacitance against the bilayer area was strictly linear extrapolating to zero capacitance in the limit of zero area. The value of the specific capacitance, *C*, of the bilayer calculated from the slope, was 0.38 ± 0.02 μF/cm^2. This value is slightly less than the value of 0.40 ± 0.02 μF/cm^2 obtained for planar bilayers of similar composition (Redwood et al., 1969, 1971). Transference numbers for K^+ and Cl^- tabulated in Table I, column 1, were calculated using eq 1. The values $t^+ = 0.59 \pm 0.01$ and $t^- = 0.41 \pm 0.01$ are in excellent agreement with values obtained for bilayers formed from egg phosphatidylcholine and *n*-decane by Andreoli et al. (1967). A higher value of the cation transference number, $t_+ = 0.83 \pm 0.01$ ($t_- = 0.17 \pm 0.01$) was reported by Pagano and Thompson (1968) for the low resistance membrane formed from egg phosphatidylcholine in *n*-tetradecane, chloroform, and methanol (Table I, column 3). We have

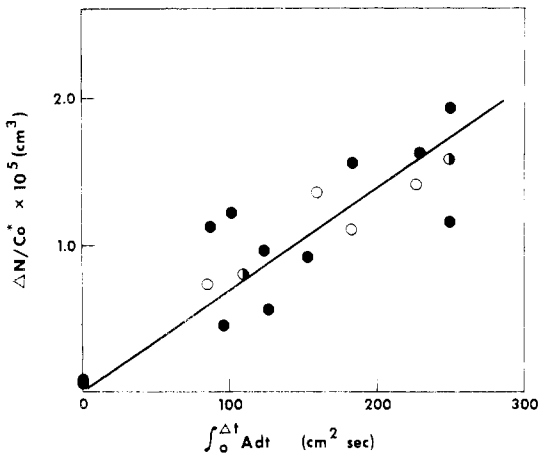

FIGURE 5: Inward ^{36}Cl flux data plotted as $\Delta N/C_0^*$ vs. $\int_0^{\Delta t} A dt$. A is the area of the bilayer membrane. The measurements were done in 0.1 M KCl at 20°. (●) open circuit; (○) the external solution 10 mV positively polarized; (◑) the external solution 10 mV negatively polarized.

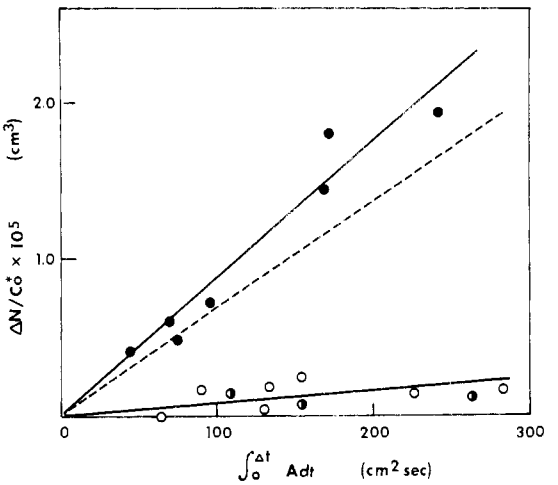

FIGURE 6: Effect of substitution of nitrate for unlabeled chloride on ^{36}Cl inward flux. (●) External and internal solutions are 0.1 M KNO_3 and 0.1 M KCl, respectively, no short circuit; (○) the external and internal solutions are 0.1 M KCl and 0.1 M KNO_3, respectively, no short circuit; (○) the external and internal solutions are 0.1 M KCl and 0.1 M KNO_3, respectively, and both solutions are kept at equipotential by an external short circuit; (- - -) least-squares fit of the data in Figure 5.

found in subsequent studies that values for cation and anion transference numbers for egg phosphatidylcholine show considerably larger day to day variations and variations among different samples than do bilayers formed from diphytanoylphosphatidylcholine. The large value as well as the variability of the cation transference number of egg phosphatidylcholine bilayers is quite possibly due to the presence of anionic oxidation products formed from the unsaturated acyl groups of this phospholipid. Diphytanoylphosphatidylcholine is, of course, not subject to autoxidation.

Permeabilities for potassium and chloride ions, k_K^{ion} and k_{Cl}^{ion}, were calculated from eq 2 using the values of the transference numbers and membrane conductance shown in Table I, and $C_i = 10^{-4}$mol/ml. In these calculations K^+ and Cl^- were assumed to be responsible for the total bilayer conductance. This assumption seems reasonable since contributions to the conductance by H^+ and OH^- are negligible in this type of bilayer as evidenced by the minimal dependence of conductance on pH reported by Redwood et al. (1971).

Isotopic Chloride Flux. The data for the inward chloride flux measured with ^{36}Cl at 20° in 0.1 M KCl solution are shown in Figure 5. The solid circles represent data obtained in the absence of the applied trans-bilayer voltage. The open and semisolid circles represent the chloride flux in the presence of the electrical field of ±10 mV applied externally. The data show no apparent dependence of the chloride permeability on the applied electrical field. The chloride permeability coefficient, k_{Cl}, obtained from the slope of the line in Figure 5 is 6.8×10^{-8} cm/sec. This value is more than 10^4-fold larger than the value of k_{Cl}^{ion} calculated from the electrical measurements under identical conditions (Table I, column 1). This result together with the fact that the ^{36}Cl flux was independent of an applied external field indicates that the major component of the isotopic chloride flux cannot be due to permeation through the bilayer of chloride bearing a net charge. This same conclusion was reached by Pagano and Thompson (1968) for the low resistance bilayer formed from egg phosphatidylcholine and the mixed solvent which had k_{Cl}/k_{Cl^-} equal to about 10^2.

The dependence of the flux of ^{36}Cl on the absence of Cl^- on either the cis or trans sides of the bilayer is shown in Figure 6. In this series of experiments NO_3^- was substituted for Cl^- in order to maintain a constant anion concentration. This was necessary since the semispherical bilayer proved to be mechanically unstable if the total salt concentration on one side of the membrane was reduced to zero. The dashed line in this figure, the least-squares linear fit of the flux data in Figure 5, is given for comparison. As shown by the solid circles, the flux of ^{36}Cl was not changed significantly when NO_3^- was substituted for Cl^- on the cis side (outside aqueous compartment) of the bilayer initially containing ^{36}Cl. On the other hand, the open circles show that the flux of ^{36}Cl was markedly reduced when NO_3^- was substituted for Cl^- on the trans side (inside aqueous compartment) of the bilayer intially free of ^{36}Cl. All data were obtained under conditions such that the internal and external aqueous compartments were not externally short circuited. These results are in qualitative agreement with the findings of Pagano and Thompson (1968) for egg phosphatidylcholine-mixed solvent bilayers. In their experiments, inward chloride flux was reduced 10^3-fold when nitrate was substituted for chloride on the trans side of the spherical bilayer. A comparison of results is made in Table II. The fact that cis substitution of nitrate for chloride did not reduce the flux of chloride indicates that nitrate per se did not alter the permeability of the bilayer to ^{36}Cl. It may therefore be concluded that the inward flux of chloride is coupled to an outward flux of chloride.

The data points indicated by semisolid circles in Figure 6 were obtained with NO_3^- replacing Cl^- on the trans side and with the two aqueous compartments short circuited externally. The fact that short circuiting had no significant effect on the ^{36}Cl flux in this system clearly rules out the possibility that the coupling of the inward and outward fluxes of chloride is electrical. If the coupling were electrical, it should have been eliminated when both aqueous solutions were kept at equipotential by means of the external short circuit, and consequently the ^{36}Cl flux should have increased to the value indicated by the broken line in Figure 6.

The effect of thiosulfate and I^- on the ^{36}Cl flux is shown in Figure 7. The solid circles represent the inward ^{36}Cl flux

Table II: ^{36}Cl Permeability Coefficients.

Aqueous Solution Composition (mol/l.)		Permeability Coefficient, k_{Cl} (cm/sec)	
External	Internal	Semispherical[a]	Spherical[b]
0.1 KCl		6.8×10^{-8}	4.5×10^{-7}[c]
0.1 KCl, 0.01 $Na_2S_2O_3$		6.0×10^{-8}	
0.1 KCl, 0.001–0.1 KI		$<2 \times 10^{-9}$	
0.1 KCl	0.1 KNO_3	$<6 \times 10^{-9}$	4.2×10^{-10}[d]
0.1 KNO_3	0.1 KCl	8.4×10^{-8}	

[a] Diphytanoylphosphatidylcholine in *n*-decane. [b] Egg phosphatidylcholine in *n*-tetradecane, chloroform, and methanol (Pagano and Thompson, 1968). [c] Measured in 0.2 *M* NaCl solution at 30°. [d] Measured in the system of 0.2 *M* NaCl in the external solution and 0.2 *M* $NaNO_3$ in the internal solution at 30°.

in 0.1 *M* KCl with 0.01 *M* $Na_2S_2O_3$ added. For comparison, the dashed line is the least-squares fit of the flux data in Figure 5. Previously Gutknecht and coworkers (1972) found that the bromide permeability was reduced by one order of magnitude by addition of thiosulfate, a reducing agent. On the basis of their results, they concluded the principle flux of bromide in their system was, in fact, a flux of Br_2. The data in Figure 7, however, show no significant effect of thiosulfate on the ^{36}Cl flux. This result rules out the possibility that the chloride flux is a flux of molecular chlorine. The chloride flux was, however, substantially reduced when iodide was added to the aqueous phases, even at concentrations as low as 0.001 *M*. This is shown by data plotted in Figure 7 as open and semishaded circles. A summary of the ^{36}Cl flux measurements in the semispherical bilayer system are presented in Table II. The results of Pagano and Thompson (1968) for the egg phosphatidylcholine-mixed solvent system are also presented for comparison.

Mechanism of ^{36}Cl Permeation. The results obtained with a high resistance semispherical bilayer are in qualitative agreement with those reported earlier by Pagano and Thompson (1968) for spherical bilayers formed from a chloroform–methanol solution on egg phosphatidylcholine and *n*-tetradecane. Intrepretation of the data obtained in the latter system was complicated by two factors. (1) It is well known that oxidation of the unsaturated acyl chains of egg phosphatidylcholine bilayers is difficult to prevent. The presence in the bilayer of oxidation products in small amounts could provide the mechanism for enhanced ^{36}Cl permeation. The fact that semispherical bilayers formed from the completely saturated, nonoxidizable diphytanoylphosphatidylcholine showed a similar enhancement in ^{36}Cl permeation rules out the involvement in the permeation process of acyl chain oxidation products. (2) The presence of both chloroform and methanol in the bilayer could conceivably modulate ^{36}Cl permeation. More reasonable, however, is the possibility that the enhanced electrically silent chloride permeability may be due to the presence in the bilayer of the spontaneous oxidation products of chloroform. The similarity of results obtained with the semispherical bilayer system which contained no chloroform or methanol eliminated these possibilities.

The large difference between the k_{Cl} determined isotopically and k_{Cl}^{ion} determined electrically indicates that the flux of ^{36}Cl cannot be due to the simple permeation of Cl^-. This conclusion is consistent with the observation that the flux was independent of the electric field applied across the bilayer. The fact that ^{36}Cl permeation was unaffected by thiosulfate, a reducing agent, in the aqueous phases rules

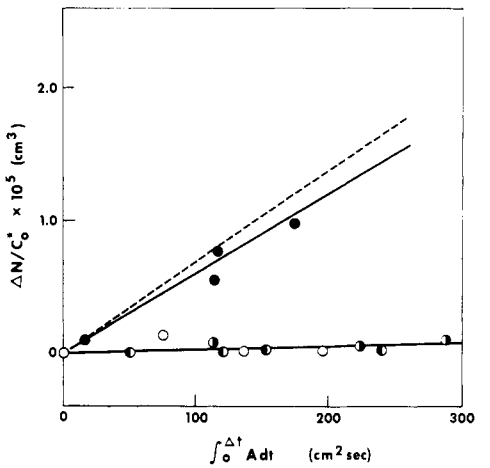

FIGURE 7: Effect of thiosulfate and iodide on the chloride flux. Aqueous phase composition: (●) 0.1 *M* KCl plus 0.01 *M* $Na_2S_2O_3$; (○) 0.1 *M* KCl plus 0.001 *M* KI; (◑) 0.1 *M* KCl plus 0.01 *M* KI; (◐) 0.1 *M* KCl plus 0.1 *M* KI; (- - -) least-squares fit of the data in Figure 5.

out the possibility that a major component of the ^{36}Cl flux is due to Cl_2. This conclusion is supported by the observation that the inward flux of ^{36}Cl was independent of the concentration of unlabeled chloride on the cis side of the bilayer. If the flux of ^{36}Cl were in fact a flux of ^{36}Cl-Cl, then the measured flux would be dependent on the concentration of Cl^-.

These facts make it certain that ^{36}Cl moves across the bilayer as a neutral complex species which is not Cl_2. Two simple types of carrier mediated mechanisms are conceivable. The first is a positively charged carrier present in the aqueous phases which moves across the bilayer as an ion pair with Cl^-. The second mechanism requires a membrane bound carrier which either complexes with Cl^- alone or with Cl^- and a cation.

Possible carriers of the first type are K^+, other inorganic cations such as Ca^{2+} or Fe^{3+}, and H^+. Ion pair formation with K^+ was ruled out in earlier experiments (Pagano and Thompson, 1968). If this were the case, then k_K inferred from ^{22}Na flux experiments and k_{Cl} would be expected to be nearly equal. In fact k_{Cl} was larger than k_{Na} by more than two orders of magnitude in the egg phosphatidylcholine-mixed solvent system (Pagano and Thompson, 1968). In addition, the reduction in the ^{36}Cl inward flux by the substitution of NO_3^- for Cl^- on the trans side of the bilayer is inconsistent with a permeation mechanism involving ion pair formation between Cl^- and K^+. These results also

make it unlikely that the permanent complex is an ion pair formed between Cl^- and some other trace inorganic cation. Bangham (1972), however, has suggested that ^{36}Cl permeates liposome bilayer membranes as the neutral species HCl. Recently, Kornberg and coworkers (1972) found that in egg phosphatidylcholine vesicles an inside-outside concentration gradient of chloride induced a hydrogen ion gradient across the bilayer. The hydrogen ion distribution in their vesicle system was determined using the spin-label tempotartrate as an indicator. They concluded that a flux of molecular HCl existed in their system. Our results may also be explained in part on the basis of a flux of molecular $H^{36}Cl$, since if this is the case, the ^{36}Cl flux would not be expected to contribute to the elctrical conductance of the bilayer and would not depend on an external electric field. Likewise, the reduction of the inward flux of ^{36}Cl by substitution of NO_3^- for Cl^- on the trans side of the bilayer would be expected, if the permeant species were $H^{36}Cl$. This would follow since in the absence of a buffer in the aqueous compartment, which was the condition under which the experiments reported in this paper were done, initial HCl permeation into the semispherical bilayer would generate a trans bilayer pH gradient which would quickly reduce the HCl flux to zero. However, under these conditions an electrical short circuit should bring both sides of the bilayer to the same potential and restore the ^{36}Cl flux. As shown in Figure 6, however, this did not occur. It thus seems unlikely that a major component of the ^{36}Cl flux can in fact be $H^{36}Cl$.

As candidates for a membrane-bound chloride carrier, two possibilities are apparent; the first is a trace impurity present in the bilayer, the second is the phospholipid. Although it is virtually impossible to rule out experimentally the involvement of a trace impurity, it seems unlikely that impurities were present in the system. The synthetic diphytanoylphosphatidylcholine is very stable to oxidation. All preparations were chromatographed on thin-layer silicic acid prior to use and exhibited a single component. The *n*-decane (99.5%, olefin free) was also chromatographed prior to use on an activated alumina column.

Hanai and coworkers (1965a,b) some years ago suggested that the electrical conductivity of phospholipid bilayers may be due to an ion-phospholipid carrier mechanism. An exchange diffusion of ^{36}Cl could be mediated by a cation-chloride complex. Although the first possibility may be ruled out since it would be expected to give use to a large cation exchange flux, which was not observed (Pagano and Thompson, 1968), Cl-phospholipid or HCl-phospholipid carrier complexes are compatible with all the experimental evidence. If the Cl-phospholipid carrier exists, the quaternary ammonium group of phosphatidylcholine is presumably associated with chloride during transit across the membrane. If HCl-phospholipid carrier exists, then in addition to the binding of chloride by the quaternary ammonium group of phosphatidylcholine, there is concomitant hydrogen ion binding to the phosphate group. The reduction of chloride permeability obtained upon addition of iodide to the system is consistent with chloride binding to the quaternary ammonium group. Since the binding constant of this group for I^- is much larger than it is for Cl^- (DeGeiso et al., 1954), added I^- in competition with Cl^- for the binding site would be expected to reduce the ^{36}Cl flux. Recent proton nuclear magnetic resonance studies have in fact shown that I^- interacts more strongly than Cl^- with the quaternary ammonium group of phosphatidylcholine in bilayers (Jendrasiak, 1972).

The strong possibility that a chloride-phospholipid complex is the transport species giving rise to the large ^{36}Cl flux was first suggested by Pagano and Thompson (1968). Recently Kornberg and McConnell (1971) have suggested that this flux may be associated with the trans membrane "flip-flop" of phosphatidylcholine observed by these workers in vesicle systems using electron spin resonance spin-label techniques. In the following paper reporting studies carried out on similar phospholipid vesicles, we show that the experimentally determined flux of ^{36}Cl in this vesicle system agrees well with the value calculated from the "flip-flop" parameters (Toyoshima and Thompson, 1975).

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