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The Effect of the Polar Moiety of Lipids on the Ion Permeability of Bilayer Membranes

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Summary. Bilayer membranes were prepared with the negatively charged lipids phosphatidylglycerol and diphosphatidylglycerol, the positively charged lipid lysyl phosphatidylglycerol, the zwitterionic lipid phosphatidylethanolamine, and an uncharged glycolipid, diglucosyldiglyceride, all isolated from gram-positive bacteria. Bilayer membranes of all these lipids manifested specific resistances of 10^7 to 10^9 Ω cm^2 and capacitances of 0.3 to 0.4 μF cm^{-2}. The membrane potentials of these bilayers were measured as a function of the sodium chloride, potassium chloride, and hydrogen chloride transmembrane concentration gradients (0.01 to 0.10 M) and were found to be linear with the logarithm of the salt activity gradients. Membranes made from lysyl phosphatidylglycerol (one net positive charge) were almost completely chloride selective, whereas membranes from phosphatidylglycerol and diphosphatidylglycerol (one and two net negative charges, respectively) were highly cation selective. Membranes prepared with either diglucosyldiglyceride or phosphatidylethanolamine showed only slight cation selectivity. These findings indicate that the charge on the polar head group of membrane lipids plays an important role in controlling the ion-selective permeability of the bilayer.

Following the original report by Mueller, Rudin, Tien, and Westcott (1962 *a*, *b*) on the preparation of thin films of brain lipids, numerous workers have investigated the physical properties of these structures (*see* reviews by Rothfield & Finkelstein, 1968; by Tien & Diana, 1968; and by Henn & Thompson, 1969). Measurements of thickness, electrical resistance and capacitance, surface tension, and water permeability suggest that these films form bilayer structures similar to the bilayer structure proposed by Danielli and Davson (1935, 1936) and by Robertson (1959) for natural membranes.

A number of different amphipathic molecules, charged and uncharged, have been used successfully to make stable bilayer membranes. Under conditions in which the membrane separates two aqueous solutions of different

ionic concentration, a diffusion potential may be observed. Since it has been shown for other types of model membranes that fixed charges on the membrane surface are very important in determining the magnitude and sign of diffusion potentials (Teorell, 1953), it was of interest to examine if membranes made from naturally occurring lipids behave in a similar manner.

Moreover, it also was of particular interest to investigate the relationship of such phenomena to certain characteristics of natural systems. For instance, it has been observed that some gram-positive bacteria alter the ratio of negatively to positively charged lipids in their cell membrane upon a change in the pH of the growth medium (Houtsmuller & van Deenen, 1964, 1965; Op den Kamp, van Iterson, & van Deenen, 1967; Op den Kamp, Redai, & van Deenen, 1969).

The major negatively charged lipids in *Staphylococcus aureus* are phosphatidylglycerol (PG) and diphosphatidylglycerol (DiPG), whereas the major positively charged one is lysyl phosphatidylglycerol (LysPG). PG and DiPG are the dominant charged lipids at pH 7. At pH 5 the proportion of PG decreases markedly, whereas that of LysPG increases (Houtsmuller & van Deenen, 1964, 1965). In this study, bilayer membranes were formed from the above-mentioned charged lipids and the major uncharged lipid of *S. aureus*, diglucosyldiglyceride (DiGluDiGly). In addition, membranes were prepared with the zwitterionic lipid phosphatidylethanolamine (PE) isolated from *Bacillus megaterium* and *Escherichia coli* (for structure of lipids, *see* Fig. 1). The resistance and capacitance of the membranes, as well as their relative permeability to sodium, potassium, hydrogen, and chloride ions, were determined.

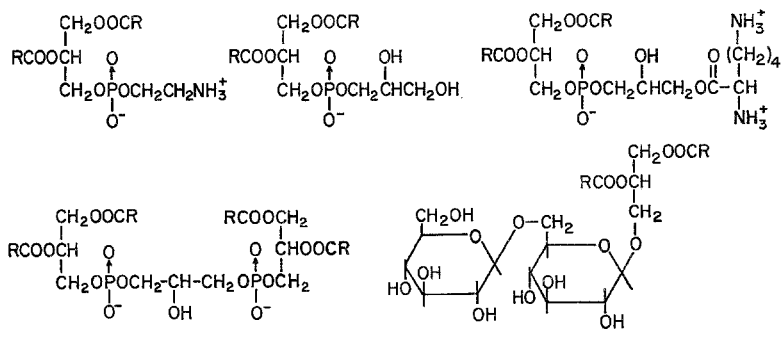

Fig. 1. Structures of the lipids used for preparation of membranes. *Top row*, from left to right: phosphatidylethanolamine (PE), phosphatidylglycerol (PG), lysyl phosphatidylglycerol (LysPG). *Bottom row*, from left to right: diphosphatidylglycerol (DiPG), diglucosyldiglyceride (DiGluDiGly). *R* represents the fatty acyl groups

Materials and Methods

Materials

Distilled water was passed over a mixed-bed ion-exchange resin (Barnstead) and redistilled through an all-glass still. Analytical reagent grade preparations of NaCl and KCl were recrystallized from a solution of 1 mM ethylenediaminetetraacetate (EDTA). With the exception of decane which was passed over an alumina column and redistilled, all organic solvents (reagent grade) were used without further treatment. Cholesterol (Sigma) was recrystallized twice from absolute ethanol.

Methods

Analytical Thin Layer Chromatography (TLC). Plates for analytical TLC were prepared from silica gel G (E. Merck) suspended in 0.01 M Na_2CO_3 (Stahl, 1962). Plates were developed in chloroform-methanol-water (65:25:4) (Wagner, Hörhammer, & Wolff, 1961). Approximately 50 to 100 μg of lipid were applied for analytical purposes.

Spots were detected using iodine vapor as a general lipid indicator, molybdenum blue reagent (Dittmer & Lester, 1964) for phospholipids (PE, PG, LysPG, DiPG), the ninhydrin reaction for amino group-containing lipids (PE, LysPG), and an aniline-diphenylamine spray for glycolipids (Harris & MacWilliams, 1954).

The R_f values in the above system for these lipids were as follows: LysPG, 0.20; PG, PE, 0.37; DiGluDiGly, 0.56; and DiPG, 0.80.

Preparative TLC. A 1-kg amount of silica gel H (E. Merck) was washed batchwise with 8 liters of chloroform-methanol-formic acid (1:2:1) followed by 4 liters of water, and then dried at 110 °C for 48 hr. The slurry of gel in the appropriate solution (*see* below) was suspended in a Waring Blendor for 5 min. The gel used for preparation of LysPG was suspended in water (approximately 48 ml water/30 g silica gel H), whereas that for PE and DiPG purification was prepared with 0.01 M Na_2CO_3 (approximately 52 ml/30 g silica gel H). The plate thickness was 0.75 mm. Samples were applied in streaks, and the plates were developed in chloroform-methanol-water (65:25:4). Small side strips of the chromatogram were sprayed with phospholipid stain in order to detect the phospholipid. The main bands were scraped off, and the lipid was eluted with chloroform-methanol (1:1).

Purification of Lipids. Crude lipids were isolated by chloroform-methanol (2:1) extraction (Folch, Lees, & Sloane-Stanley, 1957) of *S. aureus* (strain 655 or 63), *B. megaterium* (strain KM), or *E. coli* (strain B) grown in rich media and harvested in the late logarithmic phase of growth. After extraction of the cells for 12 hr and filtration to remove the insoluble residue, the chloroform extract was washed with one-fifth volume of 0.9 % NaCl, pH 2. Initial fractionation was achieved on a diethylaminoethanol (DEAE)-cellulose column according to Rouser, Kritchevsky, Heller, and Lieber (1963). Four fractions were collected upon elution with the following solvents: (1) chloroform-methanol 2:1; (2) methanol; (3) 0.01 M ammonium acetate in methanol; and (4) 0.05 M ammonium acetate in chloroform-methanol 4:1 containing 2 % (v/v) concentrated NH_4OH.

DiGluDiGly was prepared from a sample of fraction 1 of the DEAE-cellulose column. The crude glycolipid, dissolved in chloroform, was applied to a silicic acid column (Unisil 200 – 325 mesh; Clarkson Chemical Co.). The column was washed with chloroform and then eluted with acetone. Analytical TLC of the latter fraction revealed only one spot which reacted with iodine and the aniline-diphenylamine stain, but not with the phospholipid or ninhydrin stains. The behavior of this spot on DEAE-cellulose, silicic acid, and staining with the aniline spray proved it to be a glycolipid. It was the major

glycolipid from *S. aureus*, the structure of which has been determined to be 1-(0-β-D-glucopyranosyl-(1→6)-0-β-D-glucopyranosyl)-2, 3 diglyceride (Shaw & Baddiley, 1968).

For the preparation of LysPG, a small silicic acid column (1 g Unisil/200 mg lipid) was used. A second sample of fraction 1 of the DEAE-cellulose was evaporated to dryness, redissolved in chloroform, and applied to the silicic acid column. The column was washed with 100 ml of chloroform, and then with 200 ml acetone/g Unisil. The LysPG-rich fraction was subsequently eluted with chloroform-methanol (1:1) containing 4 % water. Further purification was achieved by preparative TLC on silica gel H plates made with water. Traces of PG and other minor contaminants were removed by elution with chloroform-methanol (2:1) through a small DEAE-cellulose column, and then by acetone precipitation from anhydrous ether. Analytical TLC of the final product showed only one spot when tested with iodine, phospholipid, and ninhydrin spray. Acid hydrolysis in 6 N HCl for 24 hr at 100 °C and subsequent analysis on an amino acid analyzer revealed a single peak for lysine. The lysine:phosphate ratio was 1.06.

PG was isolated from fraction 3 of the DEAE-cellulose column. TLC revealed only one spot with iodine and phospholipid spray; no reaction was observed with ninhydrin.

DiPG was obtained from fraction 4 of the DEAE-cellulose column. Further purification was achieved by preparative TLC. A green pigment which had the same R_f as DiPG on TLC was removed by passage over a silicic acid column. The sample was applied in chloroform, and the column was then eluted with one bed volume of chloroform and subsequently by acetone. The acetone fraction, which contained the green pigment, was discarded, and DiPG was then eluted with chloroform-methanol (2:1). DiPG was identified by co-chromatography on TLC with a sample of beef heart DiPG (Applied Science Labs.).

PE was isolated from the lipid extracts of *B. megaterium* and *E. coli* by chromatography on a DEAE-cellulose column. The column was eluted sequentially with chloroform-methanol 9:1; chloroform-methanol 7:1; and chloroform-methanol 2:1. PE, which was eluted with chloroform-methanol 2:1, was further purified by preparative TLC. The final product showed only one spot by iodine, phospholipid, and ninhydrin spray. The bacterial PE co-chromatographed with beef heart PE (Applied Science Labs.).

Preparation of Membrane Bilayers. The cell used for the formation of bilayers was similar in design to that of Andreoli, Bangham, and Tosteson (1967). It consisted of two lucite chambers, an open one 1.5 × 3.8 × 3.0 cm (depth × length × height) and a closed cylindrical one with a radius of 0.7 cm and a depth of 1.2 cm, separated by a plexiglass septum (0.85-mm thick) containing a hole 2 mm in diameter. The closed chamber had two apertures at the top in addition to the hole in the septum. One aperture was used for the electrical connection, and the second was fitted with a nylon screw to adjust the volume of this chamber. The septum was sealed between the chambers by means of high-vacuum grease (Dow Corning), and excess grease and dirt were then removed with absolute ethanol and petroleum ether. Electrical connections into the chambers were made via calomel electrodes and a saturated KCl-2 % agar bridge. The open chamber was stirred by a small magnetic bar.

The cell was mounted as previously described (Huang, Wheeldon, & Thompson, 1964). The membrane-forming solution consisted of a dispersion of 1 % purified lipid and 0.4 % cholesterol in decane. Small amounts of water were added when necessary to obtain more homogeneous dispersions. The area of the hole in the septum was prepainted with the membrane-forming solution, the decane was removed under vacuum, and then the chambers were filled with the appropriate aqueous solution. The bilayer membranes were then formed by the brush technique of Mueller *et al.* (1963), and the thinning process was observed through an optical system as previously described (Huang

et al., 1964). All experiments were performed at room temperature, which varied from 24 to 26 °C.

Electrical Measurements. The electrical circuit in which the membrane is represented by its electrical equivalent is shown in Fig. 2. In Table 1, the switch modes of the circuit utilized for the various electrical measurements are shown. The closed chamber of the cell was connected to the input of an electrometer amplifier (model 201 C, E-H Research Lab., Oakland, Calif.). All connections were made with shielded cable and BNC-type connectors, and care was taken to keep the parisitic resistance of switches in the open position and of capacitors on the high impedance side above 10^{13} Ω. The DC-output

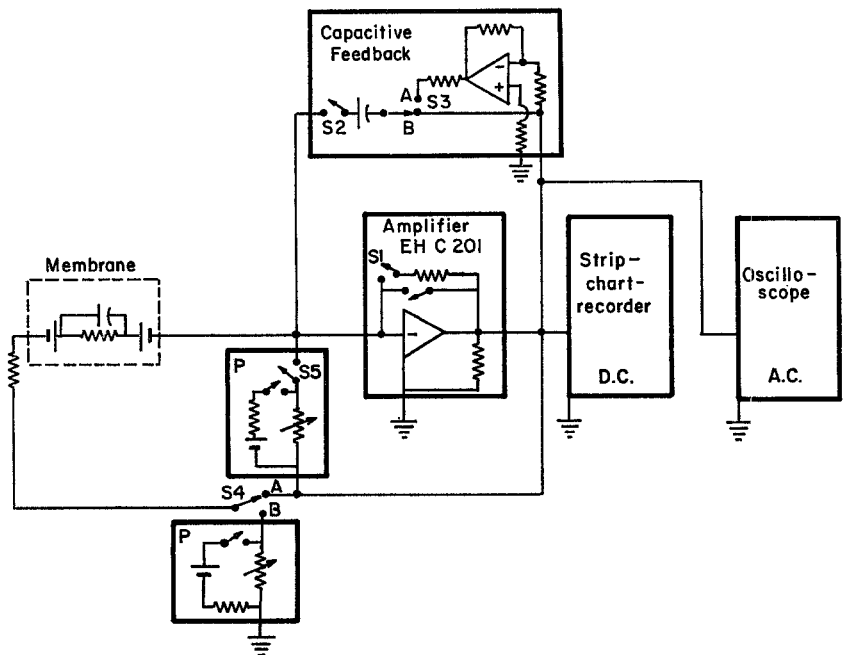

Fig. 2. Electrical circuit for measurement of the membrane potentials. The membrane is represented by its electrical equivalent. *See* Table 1 for a description of the switch positions

Table 1. *Switch positions used in electrical measurements* [a]

Measurement	Switch position				
	S 1	*S* 2	*S* 3	*S* 4	*S* 5
Potential	Open	Open	—	*A*	Open
Potential with positive capacitive feedback	Open	Closed	*A*	*A*	Open
DC resistance	Closed	Closed	*B*	*B*	Open
DC capacitance, charging of the membrane capacitor	Closed	Open	—	*A*	Closed

[a] See Fig. 2.

of the electrometer was recorded by a potentiometric recorder, the AC-output by an oscilloscope.

For measurements of the DC resistance, the open chamber was connected through a potential-divider circuit to ground, and the electrometer was used in the ammeter mode. Any 60-cps pickup was shunted by a capacitor of 2 nF. The output voltage of the electrometer was recorded as a function of stepwise changes in the voltage across the potentiometer (± 20 mV).

The membrane resistance was calculated by means of the following equation:

$$R_m = \frac{-R_f V_{in}}{b V_{out}},$$

where R_m = membrane resistance, R_f = feedback resistance, V_{in} = voltage across the potentiometer, V_{out} = output voltage of amplifier, and b = feedback ratio (0.9). The error due to other resistances in the circuit (electrodes, amplifier) was negligible since the membrane resistance was always at least three orders of magnitude higher than the sum of the resistances from these other components. The resistance of the system without the membrane, which was mainly the resistance of the electrodes, was about 0.2 to 0.4 MΩ. The accuracy of resistance measurements with the electrical circuit was checked by replacing the membrane with a known resistor of $10^{11} \pm 2\%$ Ω.

The membrane potential was measured under conditions of zero current. For this purpose the open chamber was connected to the output of the electrometer, which was used in the amplifier mode. The gain was set this way at unity, and the amplifier was balanced by short-circuiting the input to the output. The resistance of the amplifier was greater than 10^{13} Ω.

The potential was recorded continuously, since in this manner it was possible to distinguish between the transient and the equilibrium diffusion potentials (the steady state diffusion potentials). In order to measure the potentials of membranes with time constants ($R_m \times C_m$) of greater than 200 sec, positive capacitive feedback was used to decrease the effective time constant.

Since under the above conditions any current across the membrane would be recorded as voltage at the amplifier output, the grid current was checked and found to be about 3×10^{-15} amp, which was equivalent to only 0.3 mV when the resistance measured was 10^{11} Ω. A more significant potential error that was noted was an air-to-ground current which varied from day to day. However, this current was stable on any given day; it was minimized by maintaining a low humidity in the laboratory, by grounding all air inlets to the room, and by placing an aluminum screen around the cell. Under these conditions, the offset current was maintained between 10^{-14} and 10^{-13} amp. Despite these precautions, some very-high-resistance membranes (1 to 3×10^9 Ω cm^2) showed a low potential when identical solutions were present in both chambers. For these membranes, the initially recorded potential was taken as a base line, and errors due to a change in resistance or a drift in offset current were minimized by checking the resistance several times during the course of an experiment. In addition, the potential was remeasured upon completion of the experiment (after equalization of the salt concentration in both chambers).

In order to measure membrane potentials as a function of the salt gradient across the membrane, the bilayer film was prepared in a solution of 10 mM NaCl or KCl containing 10 μM EDTA, pH 5.1, or in 10 mM HCl. The salt concentration in the open chamber was then changed stepwise by the addition of 100 mM NaCl or KCl containing 10 μM EDTA, pH 5.1. Since one chamber of the apparatus was closed, the bilayer films were relatively insensitive to mechanical vibrations. Buffer changes in the open chamber were accomplished by the simultaneous addition of new solution and the removal of an equal volume of the bath solution. After equilibration for 1 to 2 min, the new salt

concentration was determined in a 0.3-ml sample of the solution in the open chamber. The concentration of salt was measured with a conductivity bridge, and the values were converted to activities by means of the activity coefficients reported by MacInnes (1961 *a*). The concentration of HCl was determined with a glass electrode and an Expandomatic Beckman pH meter. The reference electrode for the pH meter was the calomel electrode connected to the open chamber. Transference numbers were calculated from membrane potentials under the assumption that only the two ions of highest concentration were permeable; that is, $t^{Cl^-} + t^{cation} = 1.00$. Under this assumption, the membrane potential is related to the transference number by the equation shown below (MacInnes, 1961 *b*):

$$V_m = (1 - 2t^{cation}) \frac{RT}{F} \ln \frac{a}{a_{ref}},$$

where V_m = membrane potential; t = transference number; R = gas constant; T = absolute temperature; F = Faraday equivalent; a = salt activity of the open chamber; and a_{ref} = salt activity of the closed chamber.

DC capacitance data were calculated from the time constant of the decay of an applied potential across the membrane and the effective resistance according to the following equation:

$$C_m = \tau \cdot \frac{(R_m + R_s)}{R_m \cdot R_s},$$

where C_m = membrane capacitance; τ = time constant; R_m = membrane resistance; and R_s = shunt resistance.

Results

General Properties of the Membranes

Membranes prepared from PG, LysPG, DiPG, DiGluDiGly, and PE were all mechanically stable for at least 2 hr in NaCl, KCl, and HCl solutions, except for DiPG, the membranes of which were unstable in HCl. The overall thinning process was similar to that described for phosphatidylcholine (Huang *et al.*, 1964), and the time necessary to reach the "secondary black" stage for the total hole area ranged from 1 to 2 min for PE membranes up to 10 to 30 min for the other lipids.[1]

The specific capacitances for the different lipid membranes ranged from 0.2 to 0.5 μF cm^{-2} (Table 2), a finding that indicates a bilayer thickness of 50 to 100 A (Hanai, Haydon, & Taylor, 1965). All the membranes showed high specific resistances varying from 10^7 to 10^9 Ω cm^2.[2] These values,

1 Membranes could also be formed from solutions lacking cholesterol, but usually these membranes showed lower stability and lower resistance than those containing cholesterol.

2 In preliminary experiments, LysPG membranes showed specific resistances of 10^5 to 10^7 Ω cm^2. In the experiments, reported here, chlorinated silver electrodes were in direct contact with the aqueous solutions surrounding the bilayer films. Resistances comparable to other lipids were obtained when KCl bridges were placed between the electrodes and the chambers, and highly purified water and salt solutions containing 10 μM EDTA were used. Commercial triple-distilled water also lowered the resistance of LysPG membranes. No such effect was observed for PG, DiPG, or DiGluDiGly.

Table 2. *Electrical properties of the lipid bilayers*

Lipid	DC resistance (Ω cm^2)	DC capacitance (μF cm^{-2})
LysPG	$10^7 - 9 \times 10^8$	0.33 – 0.38
PG	$10^8 - 2 \times 10^9$	0.32 – 0.41
DiPG	$5 \times 10^8 - 3 \times 10^9$	0.23 – 0.38
DiGluDiGly	$10^8 - 2 \times 10^9$	0.35 – 0.46
PE	$3 \times 10^7 - 2 \times 10^9$	0.28 – 0.45

shown in Table 2, are comparable with those reported by others using the lipid-decane system with mammalian lipids (Hanai *et al.*, 1965; Andreoli *et al.*, 1967). No correlation between the magnitude of the membrane potential (*see* below) and the resistance of the membrane was observed except in the case of PE membranes.

The Equilibrium Diffusion Potential

The total potential differences between the two chambers were measured as a function of the salt gradient across the membrane.

Initially, the concentration of NaCl, KCl, or HCl was 10 mM in both chambers. The closed chamber was taken as reference, and potentials were expressed with respect to this side. The salt concentration of the open chamber was then increased stepwise to a final concentration of 100 mM and subsequently decreased. Two potential changes could very often be distinguished after an increase or decrease in the salt concentration: a rapid transient one caused by a change in the boundary potential due to the altered ionic strength, and a slow one resulting from establishment of a new equilibrium diffusion potential. Fig. 3 (bottom recording) shows an example of a potential recording with a PG membrane. In this example, the magnitudes of the transient potential and of the equilibrium potential were nearly equal, concealing the transient character of the fast potential change after an increase in salt concentration. However, the existence of the transient potential became obvious when the rise time of the potential resulting from a change in salt concentration was compared with that observed after the membrane had been short-circuited.

At least two equilibrium potential values were obtained from each membrane[3], and these were then plotted vs. the logarithm of the ratio of salt

3 The asymmetry potentials of the calomel electrodes and liquid junctions measured in the absence of a membrane and with the same solution in both chambers ranged between 1 and 5 mV. However, this potential remained constant during experiments, so that the membrane potential could be corrected for this value. Differences in liquid

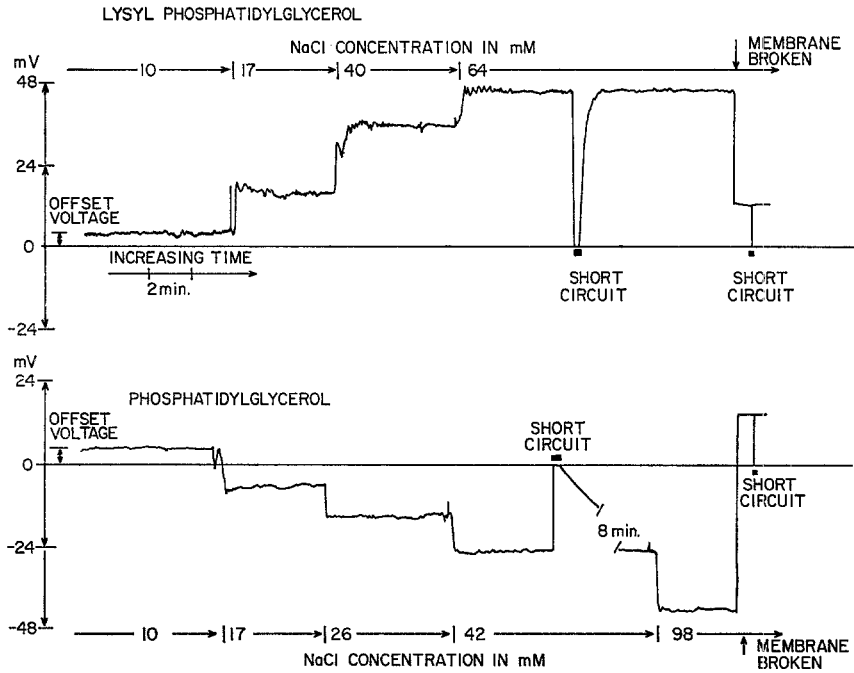

Fig. 3. Tracings of membrane potentials as a function of salt gradients across bilayers of lysyl phosphatidylglycerol and phosphatidylglycerol. The bilayer membranes are formed in an aqueous solution of 10 mM NaCl, 10 μM EDTA, pH 5.1. The NaCl concentration is increased in one chamber to the value indicated in the figure and maintained at 10 mM in the reference chamber. The initial offset voltage is measured when equal salt concentrations are in both chambers. The duration of external short circuit is indicated by black bars. The voltage measured after breakage of the membrane represents the diffusion potential of the NaCl gradient at the liquid junction

concentration in the two chambers, $\left(\log\frac{a}{a_{\text{ref}}}\right)$. As shown in Figs. 4, 5, and 6, for most membranes the potential was a linear function of $\log\frac{a}{a_{\text{ref}}}$ which indicated that the equilibrium potential represented a diffusion potential. Deviations from linearity were observed with a few membranes, especially at osmotic pressure differences higher than 0.1 osm. The range of potentials observed per 10-fold difference in salt activity from different membranes is summarized in Table 3[3]. The two negatively charged lipids consistently showed a cation diffusion potential, and, in fact, most PG membranes showed a potential which approached the theoretically maximum

junction potentials when a salt gradient was present were negligible, since the measured diffusion potentials in water after breakage of the membrane were always within 1 mV of those calculated from the known values of the transference numbers of the appropriate salt or HCl (MacInnes, 1961*c*).

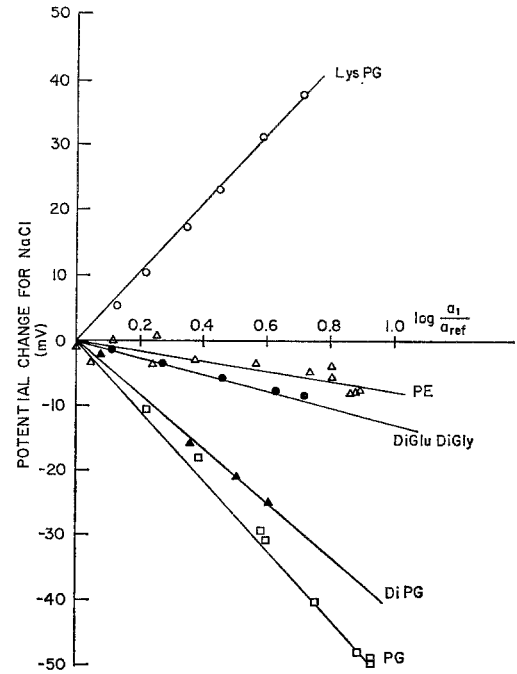

Fig. 4. Membrane potential as a function of the log of a NaCl concentration gradient across the bilayer. Values are obtained from a single membrane for each lipid

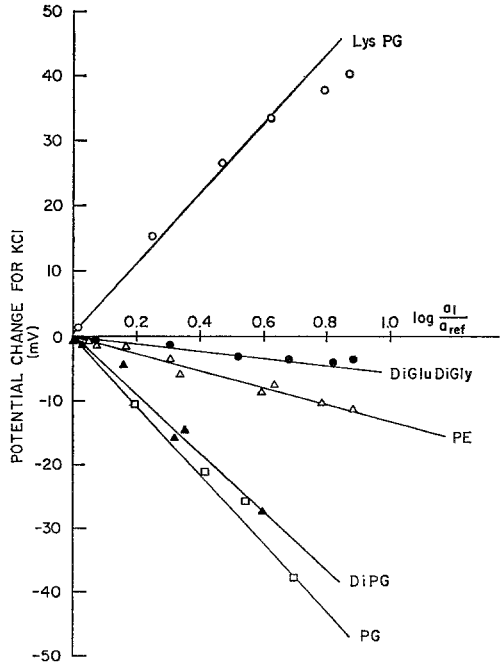

Fig. 5. Membrane potential as a function of the log of a KCl concentration gradient across the bilayer. Values are obtained from a single membrane for each lipid

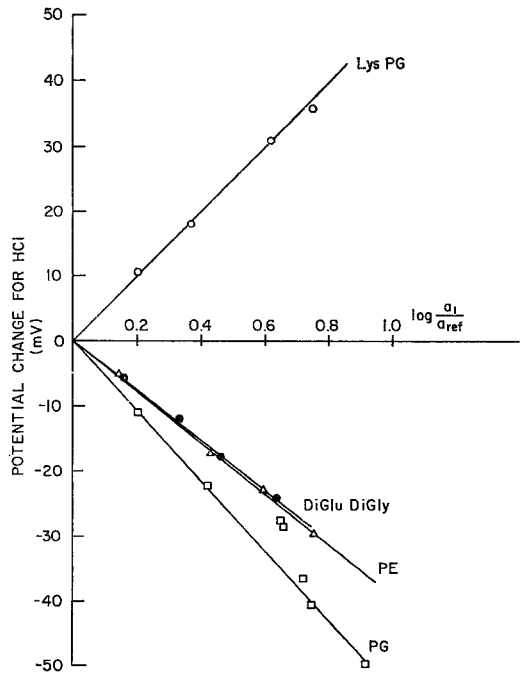

Fig. 6. Membrane potential as a function of the log of a HCl concentration gradient across the bilayer ($-\Delta$ pH). Values are obtained from a single membrane for each lipid

Table 3. *Equilibrium diffusion potentials of lipid bilayers*

Lipid	Source	Total charge	Net charge	Change in equilibrium potential per 10-fold increase in salt activity [a] (mV)		
				NaCl	KCl	HCl
LysPG[b]	*S. aureus*	++−	+	+52 to +54	+47 to +55	+48 to +49
PG[b]	*S. aureus*	−	−	−27 to −54	−39 to −54	−47 to −54
DiPG[b]	*S. aureus*	−−	−−	−27 to −43	−19 to −44	−
DiGluDiGly[b]	*S. aureus*	0	0	−3 to −13	−6	−38 to −39
PE[b]	*B. megaterium*	+−	0	+7 to −33	+30 to −19	−35 to −38
PE[c]	*B. megaterium*	+−	0	−3 to −7	−6 to −13	−
PE[c]	*E. coli*	+−	0	−6	−10 to −14	−25 to −35

[a] Individual values for the potential were calculated from the slope of the plots of Δ mV vs. $\log \frac{a}{a_{ref}}$ (*see* Figs. 4–6).

[b] 1% lipid, 0.4% cholesterol in decane.

[c] 1% lipid in decane.

cationic potential value of −59 mV. On the other hand, the positively charged LysPG membranes reproducibly manifested a positive potential, that is, an anionic diffusion potential. The uncharged glycolipid DiGluDiGly

Table 4. *Cationic transference numbers of lipid bilayers*

Lipid	Source	Total charge	Net charge	Cation transference number		
				NaCl	KCl	HCl
LysPG[a]	*S. aureus*	++−	+	0.05	0.04	0.09
PG[a]	*S. aureus*	−	−	0.95	0.95	0.95
DiPG[a]	*S. aureus*	−−	−−	0.86	0.87	—
DiGluDiGly[a]	*S. aureus*	0	0	0.61	0.55	0.82
PE[a]	*B. megaterium*	+−	0	—	—	0.81
PE[b]	*B. megaterium*	+−	0	0.56	0.61	—
PE[b]	*E. coli*	+−	0	0.55	0.62	0.79

[a] 1% lipid, 0.4% cholesterol in decane.
[b] 1% lipid in decane.

showed a very low cationic potential for NaCl and KCl solutions. The potential for HCl with DiGluDiGly membranes was also cationic, and, interestingly, it was considerably higher in magnitude.

One class of membranes prepared with the zwitterionic lipid PE exhibited potentials comparable to those observed with the uncharged DiGluDiGly, namely, those membranes which had a high specific resistance ($R_m \geqq 5 \times 10^8\ \Omega\,cm^2$) and were prepared without cholesterol. These membranes exhibited a low cationic membrane potential with NaCl, KCl, or HCl. Low-resistance membranes prepared from PE-decane and those made from PE-cholesterol-decane showed a wide variation of the potential, which in some instances even changed sign, although any given membrane showed a linear dependency on $\log\frac{a}{a_{ref}}$. No difference was found in the behavior of membranes prepared with PE from *E. coli* or *B. megaterium*.

From the equilibrium potential recorded for the various lipids, the transference numbers (t^+) were calculated for NaCl, KCl, and HCl. These values, shown in Table 4, were calculated for the maximum equilibrium potential observed with several membranes of the same lipid (*see* Discussion).

Although the potentials observed with a given membrane were always found to be linearly related to $\log\frac{a}{a_{ref}}$, there was some variation in the membrane potential of different membranes prepared with the same lipid. The possibility that this variation was due to fluxes of other ions present in low concentrations was considered; however, experiments with or without EDTA revealed no effect on the magnitude of the equilibrium diffusion potential. Since the variation was most pronounced with membranes showing a cationic diffusion potential, i.e., PG and DiPG, the possibility that a

Table 5. *Change in equilibrium potential per 10-fold increase in salt or proton activity* [a]

Lipid	Change (mV)	
	K^+	H^+
PG	−56.4	−0.4
DiPG	−38.5	−7.7

[a] The proton potential was measured between pH 5.1 and 5.8 in the presence of 10 mM KCl, 10 μM EDTA.

variable permeability of the membranes to protons was the cause of this phenomenon was investigated. This possibility seemed particularly likely since the pH of all the aqueous solutions was 5.1. Moreover, such behavior might be expected to lower the observed cationic potential since proton flux would be opposite in direction from that due to sodium or potassium. Permeability of the membrane to protons was tested by measuring the magnitude of a proton potential generated in the presence of excess salt. In a few experiments, the potential was first measured as a function of the KCl concentration, and then the concentration of the salt in the open chamber was restored to the original concentration of 10 mM. The potential was then recorded as a function of pH changes around pH 5.1. Proton potentials ranging from 0.4 to 22 mV/pH unit were found. Table 5 gives two examples of potentials measured in this manner.

Although not of direct concern in this study, the rapid transient potential ascribed to changes in boundary potential was of interest. The two phases of potential change could be observed especially well in PE and DiPG membranes for which the equilibrium potential was usually less than the transient boundary potential difference between the two sides. The highly charged membranes of PG, DiPG, and LysPG showed fast changes which were linear with $\log\frac{a}{a_{\text{ref}}}$ and approached the Nernst potential in magnitude. PE membranes showed a fast change between −15 and −20 mV per unit of $\log\frac{a}{a_{\text{ref}}}$, but the change for DiGluDiGly was too small to be measured.

Discussion

The aim of this study was to investigate the possible relationship between the charge of the polar head group of lipid bilayers and the relative permeability of the membrane bilayer to cations and anions, which is most readily expressed in terms of the transference number. Thus a cationic

transference number (t^+) greater than 0.5 is indicative of a cation-selective membrane, and a value below 0.5 indicates anion selectivity. A value of 0.5, of course, indicates that the membrane manifests equal permeability to cations and anions.

For this study we have utilized one positively charged lipid, two negatively charged lipids, a zwitterionic lipid, and an uncharged lipid (*see* Fig. 1 for structures). All the lipids were isolated from bacteria and, with one exception (PE from *E. coli*), contain very similar fatty acids (Kates, 1964).

The technique utilized in this study to obtain the transference numbers involved measurement of the equilibrium diffusion potential generated by the presence of unequal concentration of salts or HCl on each side of the bilayer membrane. Under the experimental conditions employed in this study, an increase in the NaCl, KCl, or HCl concentration on only one side of the membrane has three effects: (1) an increase in the ionic strength of the solution; (2) the establishment of a salt gradient across the membrane; and (3) the development of an osmotic pressure difference. In the case of HCl, it also changes the charge of the phospholipids since the phosphate group has a pK of about 2 (Papahadjopoulos, 1968).

Changes in ionic strength will affect the boundary potentials (Teorell, 1953). This change is determined by $\frac{RT}{F}\ln\frac{a}{a_{\text{ref}}}$ if the surface charge concentration is very high compared to the salt concentration. The values measured for PG, DiPG, and LysPG are very close to this theoretical number. The time required to change boundary potentials after addition of salt depends on the effectiveness of stirring, but not on the time constant of the membrane.

The equilibrium potential is determined by the permeability characteristics of the membrane to all ions present and any coupling between water flow and ion movement across the membrane (Katchalsky & Curran, 1965). Equilibrium is reached when the ionic fluxes cancel so that no net charge transport across the membrane takes place. In the absence of coupling between water and ion flow, the membrane potential is expected to depend linearly on $\log\frac{a}{a_{\text{ref}}}$. This expectation, which was confirmed in this study, thus indicates that the equilibrium potentials observed are indeed equilibrium diffusion potentials. However, since deviations were seen in some cases at higher osmotic pressure differences, a small variable coupling of water and ion flows seems possible.

It was shown that the membranes possess a variable but sometimes high permeability to protons even when present in extremely low concentrations

(10^{-5} M) in the solutions. High permeability to protons would serve to decrease the apparent Na^+ or K^+ potential of cation-selective membranes. Consequently, a decreased potential in these membranes is not an indication of increased anion permeability but rather is indicative of increased proton permeability. For this reason, the transference numbers were calculated from the maximum potential change measured, and it was assumed that in these membranes only the ions of high concentration (0.01 M) carry current.

The results obtained show that, at least for bilayer membranes of high resistance, there is a correlation between the charge on the polar head group and the relative ion permeability. Positively charged membranes are highly anion selective ($t^+ = 0.04$ to 0.09) whereas negatively charged membranes are highly cation selective ($t^+ = 0.86$ to 0.95). Membranes prepared with uncharged lipids show only very slight cation selectivity ($t^+ = 0.55$ to 0.62).

The basic question arising from the results obtained in this study is: What are the forces that result in the *differential selectivity* of several of the membranes to cations or anions? In principle, the overall selective permeability of membrane bilayers to anions or cations may be controlled by two factors: (1) repulsive or attractive forces due to the polar head group of the lipid, which would control the concentration of anions or cations at the boundary layer, and (2) a differential mobility of the cation vs. the anion across the hydrocarbon portion of the lipid bilayer.

The bilayer membranes of all the lipids tested in this study manifested high specific resistances. If these resistance values are expressed in terms of resistivity (resistance × unit area per unit length) values of 10^{14} to 10^{15} Ωcm, which are as high as those of bulk hydrocarbon liquids, are obtained. This suggests that the hydrocarbon layer of the membrane serves as the major energy barrier to the translocation of both cations and anions, even for lipids with charged polar groups. If one makes the reasonable assumption that the polar groups of uncharged DiGluDiGly and zwitterionic PE membranes do not exert an influence on the concentration of ions at the boundary region, then in this case the observed potential is determined by the ratio of the mobilities of the cation and anion across the hydrocarbon region. It is possible to approximate this ratio from the ion transference numbers ($t^+ = 0.6$, $t^- = 0.4$) obtained with the membranes prepared with these two lipids. The ratio of the cation to anion mobility estimated in this manner is only about 1.5.

In the case of membranes prepared from charged lipids, the surface-charge density must be very high under the experimental conditions employed, considering the magnitude of change in the boundary potential

observed with increases in ionic strength. Therefore, with these lipids, the boundary concentrations of cation and anion would be expected to differ by orders of magnitude. Since, as noted above, the permeability is determined by the boundary concentrations of ions as well as by their mobility across the bilayer, the surface charge should, as was experimentally observed, determine the overall ion selectivity when the ratio of mobility of the anion to the cation is close to unity.

The results of this study are consistent with those obtained on other kinds of charged membranes (Teorell, 1953), including those obtained by Andreoli *et al.* (1967), who observed higher cation selectivity in membranes made from a lipid extract of erythrocytes, which are rich in negatively charged phosphatidylserine, than in those made from phosphatidylcholine. Conversely, Szabo (1969) found anion selectivity in positively charged bilayer membranes prepared with oleoylamine. Isotope-exchange studies on liposomes revealed that the replacement of zwitterionic phosphatidylcholine by negatively charged lipids decreased the anionic and increased the cationic permeability (Papahadjopoulos & Bangham, 1967), thus demonstrating the same charge effect detected in the present study. Perhaps high proton permeability may explain the results of Henn (1967), who did not find differences in the transference numbers of sodium ion in bilayers made from phosphatidylcholine, phosphatidylethanolamine, and phosphatidylserine.

The reason for variability in ion selectivity of PE-cholesterol-decane membranes in NaCl and KCl solutions is unknown. Other investigators have made similar observations with PE-cholesterol-decane and phosphatidylcholine-tetradecane-chloroform-methanol membranes (Henn, 1967; Miyamoto, 1965). A variable orientation of the polar group at the site of ion translocation might be the source of the variability in ion selectivity. This effect could be expected to be more pronounced in PE-membranes containing cholesterol than in those lacking it, since the intermolecular bonding of PO^- and NH_3^+ groups which has been proposed by Abramson, Norton, and Katzman (1965) might be disturbed by cholesterol.

It is of interest to consider the possible significance of these findings in relation to the ion permeability properties of the membranes of gram-positive bacteria. Data on the electrical resistance of these membranes and on the relative mobility of ions across them are not available. However, it is probable that at least in *S. aureus* the lipids are responsible for most of the surface charge of the cytoplasmic membrane (Ward & Perkins, 1968). Houtsmuller & van Deenen (1964) proposed that the pH-dependent alterations of LysPG and PG in *S. aureus* are a physiological response to altered

proton concentrations in the medium which result in different membrane and/or boundary layer properties. This proposal gains support from the results of this study with a model system. Clearly, PG membranes are far more permeable to protons than to chloride ions, whereas the opposite is true for LysPG membranes. Moreover, the differences in permeability of these membranes, as well as the membranes prepared with neutral or zwitterionic lipids, compared to that of other ions investigated in this study suggest that changes in the lipid composition (i.e., changes in the polar head group) of the total lipids may profoundly affect the overall ion permeability of the bacterial cytoplasmic membrane.

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