The Effect of Surface Charge on the Voltage-Dependent Conductance Induced in Thin Lipid Membranes by Monazomycin

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ABSTRACT Differences in the behavior of phosphatidylethanolamine (PE) and phosphatidylylycerol (PG) thin lipid membranes treated with monazomycin are shown to be due to the negative surface charge on PG membranes. We demonstrate that shifts of the conductance-voltage (g-V) characteristic of PG films produced by changes of univalent or divalent cation concentrations result from changes of the membrane surface potential on one or both sides. In particular, if divalent cations are added to the aqueous phase not containing monazomycin, the resulting asymmetry of the surface potentials results in an intramembrane potential difference not recordable by electrodes in the bulk phases. Nevertheless, this intramembrane potential difference is “seen” by the monazomycin, and consequently the g-V characteristic is shifted along the voltage axis. These changes are accounted for by diffuse double layer theory. Thus we find it unnecessary to invoke specific binding of Mg++ or Ca++ to the negative charges of PG membranes to explain the observation that concentrations of these ions some 100-fold lower than that of the univalent cation present produce large shifts of the g-V characteristic. We suggest that analogous shifts of g-V characteristics in axons produced by changes of divalent cation concentration are also best explained by diffuse double layer theory.

In the preceding paper (Muller and Finkelstein, 1972) we described the effects of monazomycin on membranes formed from phosphatidylethanolamine (PE)—a zwitterionic lipid. Here we discuss the action of monazomycin on membranes formed from a negatively charged lipid, phosphatidylylycerol (PG). In particular, we are concerned with the steady-state properties of the system.

Although many features of PE membranes are basically unaltered in PG membranes, certain properties are completely different. We shall demonstrate that these differences are direct consequences of lipid charge and are predictable from diffuse double layer theory. Effects completely analogous
to those obtained on monazomycin-treated negatively charged membranes are also observed in biological excitable membranes.

MATERIALS AND METHODS

Except for the composition of the membrane-forming solutions, the experiments were performed as described previously (Muller and Finkelstein, 1972). Unless otherwise stated, membranes were formed from a decane solution containing 0.5% bacterial phosphatidylglycerol (PG) plus 0.5% cholesterol (molar ratio of PG to cholesterol ≈ 1:2). PG was purchased from Supelco, Inc. (Bellefonte, Pa.) and was reported to be 98% pure; it was washed with 0.01 M H₂SO₄, to remove any multivalent cations, and then extracted into ether. The fatty acid composition given was essentially the same as that of the PE used in our previous experiments. Thus, the major difference between the lipid samples is the polar head groups, PG having a net charge of -1 (Fig. 1). Cholesterol, purchased from Eastman Kodak (Rochester, N.Y.), was recrystallized twice from ethanol.

\[
\begin{align*}
R'COOCH_2 & \quad R'COOCH_2 \\
H_2C-O-P-OCH_2CH_2\text{NH}_3 & \quad H_2C-O-P-OCH_2CHCH_2\text{OH} \\
\text{PHOSPHATIDYL ETHANOLAMINE (PE)} & \quad \text{PHOSPHATIDYL GLYCEROL (PG)}
\end{align*}
\]

\( R \text{ AND } R' \text{ ARE HYDROCARBON CHAINS } \)

Figure 1. Formulas of phosphatidylethanolamine (a zwitterionic lipid) and phosphatidyglycerol (a negatively charged lipid).

RESULTS

A. General

The voltage and current clamp records obtained on monazomycin-treated PG membranes are qualitatively similar to Figs. 1 and 2 of Muller and Finkelstein (1972); any detailed differences in kinetics are irrelevant to the concerns of this paper, which are the steady-state properties of the system.

B. The Steady-State Properties of Monazomycin-Treated PG Membranes

The conductance-voltage (g-V) characteristic is similar to that of PE membranes. The log of steady-state conductance is proportional to membrane potential, i.e.

\[ g \propto e^{nqV/kT} \tag{1} \]

where \( g \) is membrane conductance, \( n \) is a constant, \( q \) is the charge on the electron, \( k \) is the Boltzmann constant, and \( T \) is the temperature in degrees Kelvin. \( n \) is 5.8 as compared to 4.4 for PE membranes. Also in agreement with data on PE membranes, conductance varies as the 5th power of monazomycin.
concentration, and the membranes display good selectivity for univalent cations. In three respects, however, the behavior of PG membranes is quite different from that of PE membranes: (a) the monazomycin concentration necessary to achieve a given conductance at a given voltage is much less (about 1/99th). Since conductance at a given voltage increases as the 5th power of the monazomycin concentration, this difference in sensitivity is staggering. (b) Symmetrical increases of uni-univalent salt concentration produce dramatic decreases in conductance at a given voltage (Fig. 2), instead of the linear increases seen with PE membranes (Fig. 5 of Muller and Finkelstein, 1972). (c) Divalent cations profoundly shift the $g-V$ characteristic (Figs. 3, 7-9), whereas they are virtually without effect on PE membranes. We shall now demonstrate that these differences are simple consequences of the negative surface charge on PG membranes.

**THEORY**

Reconciliation of the behavior of PG and PE membranes through diffuse double layer theory will be examined in this section. We shall apply diffuse double layer theory to PG membranes without much comment on the assumptions underlying the theory (see Discussion).

**A. Derivation and Solution of the Poisson-Boltzmann Equation**

If we assume that the phosphate group of PG molecules produces a negative surface charge density ($a$) on the membrane, then at equilibrium there will exist a surface potential ($\psi_0$) at the membrane-solution interface and a diffuse double layer of charge in the aqueous solution. For our purposes, the only quantity of interest is the value of $\psi_0$. To calculate this we combine the Boltzmann distribution and Poisson's equation to obtain the Poisson-Boltzmann equation, and solve it with appropriate boundary conditions.

We assume the membrane area is infinite, so that all quantities vary only on the coordinate $x$ normal to the membrane surface; we take $x = 0$ at the membrane surface. For simplicity, we also assume that the only ions of significant concentration are one univalent cation, one univalent anion, and one divalent cation, which we take for the sake of concreteness to be $K^+$, $Cl^-$, and $Ca^{++}$. At any point in solution these ions satisfy the Boltzmann distribution:

$$[K^+] = [K^+]_0 e^{-q\psi/kT} \quad (2a)$$
$$[Cl^-] = [Cl^-]_0 e^{q\psi/kT} \quad (2b)$$
$$[Ca^{++}] = [Ca^{++}]_0 e^{-2q\psi/kT} \quad (2c)$$

1 There is of course, one such interface on each side of the membrane.
2 We assume that the amount of charge within the membrane is so small that it is effectively approximated by zero; thus, there is no space-charge region within the membrane.
where $\Psi$ is the potential at any point $x$ in solution and $[i]_\infty$ is the concentration of the $i$th ion at infinity, i.e., very far from the membrane surface. The ions also satisfy Poisson's equation:

$$\frac{d^2 \Psi}{dx^2} = -\frac{4\pi \rho}{\epsilon}$$

where $\epsilon$ is the dielectric constant of water and $\rho$ is the space-charge density given by

$$\rho = q([K^+]_\infty + 2[Ca^{++}]_\infty - [Cl^-]_\infty).$$

Combining equations 2–4 we obtain the Poisson-Boltzmann equation for the present case:

$$\frac{d^2 \Psi}{dx^2} = -\frac{4\pi q}{\epsilon} ([K^+]_\infty e^{-q\psi/kT} + 2[Ca^{++}]_\infty e^{-2q\psi/kT} - [Cl^-]_\infty e^{q\psi/kT}).$$

Multiplying both sides by $d\Psi/dx$, integrating, and applying the boundary conditions $\Psi = 0$ and $d\Psi/dx = 0$ at $x = \infty$, we have

$$\left(\frac{d\Psi}{dx}\right)^2 = \frac{8\pi kT}{\epsilon} \left([K^+]_\infty e^{-q\psi/kT} + [Ca^{++}]_\infty e^{-2q\psi/kT} + [Cl^-]_\infty e^{q\psi/kT} - C\right)$$

where $C = ([K^+]_\infty + [Ca^{++}]_\infty + [Cl^-]_\infty).

Since the double layer as a whole is electrically neutral,

$$\sigma = -\int_0^\infty \rho \, dx.$$ 

Substituting for $\rho$ from equation 3, integrating, and remembering that $(d\Psi/dx)_{x=\infty} = 0$ we have

$$\sigma = -\frac{\epsilon}{4\pi} \left(\frac{d\Psi}{dx}\right)_{x=0}.$$ 

Substituting equation 8 into equation 6 (when $x = 0$) and invoking the electroneutrality condition

$$[K^+]_\infty + 2[Ca^{++}]_\infty = [Cl^-]_\infty$$

we finally have

$$\frac{2\pi \sigma^2}{\epsilon kT} = [K^+]_\infty (e^{q\psi/kT} + e^{-q\psi/kT} - 2)$$

$$+ [Ca^{++}]_\infty (2e^{q\psi/kT} + e^{-2q\psi/kT} - 3).$$
where $\Psi_0 = \Psi_{2-o}$. This is a special case of the equation previously derived by Grahame (1947). For our experiments with PG membranes the surface potential has large negative values ($\Psi_0 \ll -kT/q \approx -25 \text{ mV}$; see section B); therefore, equation 9 reduces to

$$\frac{2\pi \sigma^2}{ekT} = [K^+]_o e^{-q\Psi_0/kT} + [Ca^{++}]_o e^{-2q\Psi_0/kT}.$$  (9a)

Equation 9 relates the surface charge density ($\sigma$) to the potential ($\Psi_0$) at the membrane-solution interface and to the concentration of ions in the bulk aqueous phase. Since $\sigma$ is some constant which is experimentally determined (see Section B part 2 b), $\Psi_0$ is an implicit function of the cation concentrations. By numerical trial and error, the value of $\Psi_0$ can be determined for any specific values of $[K^+]_o$ and $[Ca^{++}]_o$.

B. The Effect of Surface Potential on the $g$-$V$ Characteristic of PG Membranes

1. Symmetrical Salt Solutions
   Our PG membranes have approximately 1 charge per 60 A² (see section B part 2 b). From equation 9 this means that $\Psi_0 \approx -180 \text{ mV}$ in 0.01 M KCl and $\approx -120 \text{ mV}$ in 0.1 M KCl. We now see why PG membranes are so sensitive to monazomycin. The concentration of any univalent cation, $i^+$, at the membrane-solution interfaces is given by the Boltzmann distribution:

$$[i^+]_o = [i^+]_a e^{-q\Psi_0/kT}.$$  (10)

In particular this is true for potassium and monazomycin (which, because of its amino group, is a univalent cation in the pH range of our experiments). For PE membranes, the interfacial concentrations of these ions are the same as in bulk solution, since $\Psi_0 \approx 0$ (because $\sigma \approx 0$). For PG membranes, however, their interfacial concentrations are much higher. In 0.01 M KCl, with $\Psi_0 \approx -180 \text{ mV}$, the interfacial concentrations of K⁺ and monazomycin⁺ are from equation 10, about $10^6$ times higher than in bulk solution. Thus, even if PG and PE membranes have the same intrinsic sensitivity to monazomycin⁴, PG will seem much more sensitive. The increase of monazomycin concentra-

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³ We are ignoring the asymmetry of monazomycin concentration. Monazomycin is present in such small amounts that it does not significantly alter $\Psi_0$, even though it is adsorbed on the membrane surface.

⁴ Since the nonpolar regions of these lipids are quite similar, it is not unreasonable to assume that they should have the same intrinsic sensitivity to monazomycin. One major difference, however, is that our PG membranes contain cholesterol whereas the PE membranes do not, and it might be thought that this causes the difference in sensitivity. However, PG membranes made without cholesterol are even more sensitive to monazomycin than those containing cholesterol. Similarly, PE membranes made with cholesterol (PE: cholesterol molar ratio of 1:2) are much less sensitive to monazomycin than plain PE membranes. Thus, when we correct for cholesterol, the difference in sensitivity is even greater than we have reported.
tion has by far the greater effect on conductance. Conductance varies linearly with (surface) concentration of K+ (as we have shown for PE membranes and will see for PG membranes). Thus a $10^3$-fold concentration increase raises membrane conductance proportionally. On the other hand, since conductance is proportional to the 5th power of monazomycin concentration, a $10^3$-fold increase in its concentration raises membrane conductance by a factor of $10^{15}$.

We also see why increases of KCl concentration drastically reduce the conductance of PG membranes (Fig. 2). From equation 9 a, if $[\text{Ca}^{++}]_\infty = 0$, a 10-fold increase in KCl concentration decreases $\Psi_0$ by about 60 mv. This reduces the interfacial concentration of monazomycin by 10-fold and hence decreases conductance by a factor of $10^5$. (Formally, substituting equation

![Figure 2: The effect of symmetrical increases of [KCl] on the steady-state $g$-$V$ characteristic of a monazomycin-treated PG membrane. The membrane was formed at room temperature in the presence of 13 mM KCl and 0.1 mM ethylenediaminetetraacetic acid (EDTA) (to insure that polyvalent cation concentration is zero). Monazomycin was then added to the front chamber, and, after approximately 20 min, curve A was obtained. The KCl concentration was then increased on both sides to the values indicated for curves B, C, and D. It required only about 1 min (the time to stir in the KCl solutions) for the new $g$-$V$ characteristic to be established. Note the large decrease in conductance (at a given voltage) with increasing [KCl]; this should be contrasted to the linear increase of conductance of a PE membrane with increasing [KCl] (Fig. 5 a of Muller and Finkelstein, 1972). Slope of lines = $e$-fold conductance change per 3.8 mv; monazomycin concentration = 1 $\mu$g/ml; membrane area = 1 mm$^2$.

$^5$"Decrease" and "increase" always refer to the absolute value of $\Psi_0$; a decrease in $\Psi_0$ means it becomes less negative.
9 a with $[\text{Ca}^{++}]_o = 0$ into equation 10 gives

$$
[i^+]_o = \frac{2\pi \sigma^2 [i^+]_o}{ekT [K^+]_o}.
$$

(11)

If $i^+$ is monazomycin+, then its surface concentration is inversely proportional to $[K^+]_o$. Note that this increase of [KCl] in solution does not alter the interfacial $[K^+]$, since the decrease in $\Psi_0$ exactly compensates for the increase of $[K^+]$ in solution. (Setting $i^+ = K^+$ in equation 11, we see that $[K^+]_o$ is invariant to $[K^+]_o$.)

Lastly, we see why symmetrical additions of divalent cations profoundly depress the conductance (Fig. 3). Divalent cations reduce $\Psi_0$; this in turn lowers the interfacial concentration of both $K^+$ and monazomycin+. Again, the reduced interfacial monazomycin concentration is by far the major reason for the lower conductance. Note in Fig. 3 that (as predicted from equation

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**Figure 3.** The effect of symmetrical increases of divalent cation concentration on the steady-state $g-V$ characteristic of a monazomycin-treated PG membrane. The membrane was formed at room temperature in 10 mM KCl with monazomycin in the front chamber. After approximately 15 min the curve labeled $[\text{Ca}^{++}] = 0$ was obtained. The CaCl$_2$ concentration was then increased on both sides to the values indicated on the curves. It required about 4 min for the new $g-V$ characteristic to be established. Note the large decrease in conductance (at a given voltage) with increasing $[\text{Ca}^{++}]$ (similar results are obtained with Mg$^{++}$); this is in contrast to the ineffectiveness of divalent cations on PE membranes. Slope of lines = $e$-fold conductance change per 5.3 mv; monazomycin concentration = 1 $\mu$g/ml; membrane area = 1 mm$^2$. 

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9 a) divalent cations need be present at only \( \frac{1}{100} \)th the KCl concentration to obtain substantial shifts of the \( g-V \) characteristic.

Since increases of KCl concentration depress conductance in PG membranes by reducing \( \Psi_0 \) (and hence lowering interfacial monazomycin concentration), they should raise conductance if \( \Psi_0 \) were somehow fixed. From equation 9 we see that \( \Psi_0 \) is determined only by divalent cations when their concentration is appreciable. (For the surface potentials of our PG films, this occurs when divalent cation concentration is greater than \( \frac{1}{100} \)th univalent ion concentration.) The surface concentration of monazomycin\(^+\) should then be unaffected by increases in KCl concentration, whereas the surface concentration of K\(^+\) will increase proportionally with KCl concentration. Under these circumstances, conductance of a PG membrane should vary linearly with KCl concentration just as for a PE membrane.\(^6\) This prediction is confirmed experimentally (Fig. 4).

In summary, the differences between PG and PE membranes in sensitivity to monazomycin and in response to symmetrical additions of salt and divalent cations result from the existence and alteration of the negative surface potential of PG membranes; i.e., the membrane can only respond to that monazomycin concentration which it "sees"—the interfacial concentration. Fig. 5 schematically summarizes the effect of KCl and divalent cation concentrations on the surface potential.

2. ASYMMETRICAL DIVALENT CATION CONCENTRATIONS Consider now the situation in which the KCl concentration is symmetrical but divalent cation is present on only one side of the membrane.\(^7\) Since there are no concentration gradients of permeant ions (K\(^+\) and Cl\(^-\)), there is no potential difference between the two solutions (as measured by electrodes which are, in practice, at "infinity"). Nevertheless, there exists a potential difference across the membrane, and hence an electric field within the membrane,\(^8\) because of the asymmetry of the two surface potentials (Fig. 6). The surface potential at each interface is calculated from equation 9 (or 9 a).\(^9\)

\(^6\) We now understand why conductance of PE membranes does not increase quite linearly with KCl concentration unless some divalent cation is present, and why divalent cation produces a small decrease in conductance. Both results are explained by a small negative surface charge on PE membranes, probably from some contaminant.

\(^7\) The following analysis does not require that divalent cation concentration be zero on one side, but only that its concentration is different on the two sides.

\(^8\) The instability of phosphatidylserine membranes with Ca\(^{++}\) present on only one side (Papahadjopoulos and Okhi, 1969) is probably due to dielectric breakdown caused by such large electric fields.

\(^9\) Equation 9 is not rigorously correct for a membrane bathed by asymmetric ionic solutions. To derive the appropriate equation to replace equation 9, equation 7 is replaced by

\[
2\sigma = -\left(\int_{-\infty}^{0} \rho \, dx + \int_{0}^{\infty} \rho \, dx\right)
\]

(7 a)

(where the first integral extends from \(-\infty\) to the left interface, and the second integral extends from the right interface to \(+\infty\), and equation 8 is replaced at each interface by the boundary condition...
Figure 4. The effect of symmetrical increases of [KCl] on the steady-state $g-V$ characteristic of a monazomycin-treated PG membrane, when the surface potentials are held constant by divalent cation. The membrane was formed at room temperature in a solution containing 0.01 M KCl and 0.1 M MgSO$_4$, with monazomycin in the front chamber. After approximately 20 min the curve labeled 0.01 M KCl was obtained. The KCl concentration was then increased on both sides to 0.077 M and the curve labeled was obtained 2 min later. Note that here, in the presence of a large concentration of divalent cation, the conductance increases (in fact, linearly) with [KCl], in contrast to the drastic decrease in conductance observed in the absence of divalent cation (Fig. 2). In the presence of large amounts of divalent cation, the response of PG membranes to [KCl] is the same as that of PE membranes (see Fig. 5 a of Muller and Finkelstein, 1972). Slope of lines = $e$-fold conductance change per 4.3 mv; monazomycin concentration = 5.5 $\mu$g/ml; membrane area = 1 mm$^2$. 
Figure 5. Diagram of the potential profiles for a PG membrane separating symmetrical salt solutions. At large (greater than 100 Å) distances from the membrane interfaces, the potential on both sides is zero. (The diagrams pertain to the open-circuit condition, i.e., no current is flowing.) The quantity of interest here and in Figs. 6 and 11 is \( \Psi_0 \), the value of the potential at the two interfaces. (In this case, with symmetrical salt solutions, \( \Psi_0 \) is the same at each interface.) In 0.01 M KCl \( \Psi_0 \) is \( -180 \) mv (A). Increasing [KCl] to 0.1 M reduced \( \Psi_0 \) by approximately 60 mv to \( -120 \) mv (B). Addition of divalent cation further reduces \( \Psi_0 \) (C). The concentration of monazomycin at the left interface is related to its concentration in the front chamber via the Boltzmann distribution. We see, therefore, that the result of symmetrically raising [KCl] or introducing divalent cation will be to reduce the intrfacial concentration of monazomycin and hence reduce the conductance at a given applied voltage. This is the basis for the shifts of the \( g-V \) characteristic in Figs. 2 and 3.
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FRONT REAR

![Diagram of potential profiles for a PG membrane separating symmetric KCl solutions with divalent cation on only one side.](image)

Figure 6. Diagram of the potential profiles for a PG membrane separating symmetric KCl solutions with divalent cation on only one side. As in Fig. 5, the potential at large distances from the membrane interfaces is zero on both sides (open-circuited condition). Thus, we record no potential difference between the front and rear chamber. Nevertheless, we see that because of the asymmetry of the surface potential, there exists a potential difference across the membrane proper and hence a field within the membrane. (For simplicity we have drawn the potential profile linear within the membrane; i.e., the field is constant.) In (A) the sign of this potential difference is such as to drive monazomycin (which is in the front compartment) out of the membrane. As far as the field that monazomycin "sees," the situation in (A) is identical to having no Ca++ present but instead passing sufficient current across the membrane to make the front compartment -60 mV with respect to the rear. This is the basis for the shifts of the g-V characteristics to the right in Fig. 7. In (B) the sign of the field is such as to drive monazomycin into the membrane. This, by itself, would shift the g-V characteristic to the left. In addition, however, because of the reduction of Ψ at the left interface, the monazomycin concentration at the interface is reduced (as occurred in Fig. 5 C). This, by itself, will shift the g-V characteristic downward, which formally is a shift to the right. As described in the text, these two effects virtually cancel each other; this is why the addition of divalent cation to the same side as monazomycin produces minimal shifts of the g-V characteristic (see Fig. 9).

\[ \sigma = -\left[ \frac{\varepsilon_w}{4\pi} \left( \frac{d\Psi}{dx} \right)_{z=+} - \frac{\varepsilon_m}{4\pi} \left( \frac{d\Psi}{dx} \right)_{z=-} \right] \]  

(8a)

where \( \varepsilon_w \) is the dielectric constant of water, \( \varepsilon_m \) is the dielectric constant within the membrane, and \( \sigma^+ \) and \( \sigma^- \) refer respectively to the aqueous side and the membrane side of the interface. At the KCl concentrations of our experiments it turns out (assuming \( \varepsilon_w \approx 80, \varepsilon_m \approx 2, \) and membrane thickness \( \approx 50 \) A), however, that equation 9 is an excellent approximation to the exact relation. For our purposes, the important point is that the surface potentials at the two interfaces are independent; i.e., to a good approximation, the introduction of divalent cation on one side of the membrane changes the surface potential only on that side and does not affect the other surface potential.
Let us first consider the case of divalent cation addition to the trans side (the solution in which monazomycin is not present). The reduction in $\Psi$ on the trans side produces a potential difference between the two interfaces (i.e., an electric field within the membrane) with the monazomycin side negative with respect to the other side (Fig. 6 a). This potential difference, although not measurable by electrodes, turns off the monazomycin system and therefore shifts the $g-V$ characteristic to the right along the voltage axis.

For example, if this intramembrane potential difference is $-20 \text{ mV}$ and we apply a potential difference of $+60 \text{ mV}$, the potential difference that monazomycin actually "sees" is only $+40 \text{ mV}$; we therefore reach the same conductance at $+60 \text{ mV}$ that obtains at $+40 \text{ mV}$ with no divalent cation on the trans side. For the surface potentials in our experiments, we predict from equation 9 that if $[Ca^{++}]_o \geq 0.01 [K^+]_o$, $\Psi_\sigma$ is linear in $\ln [Ca^{++}]_o$, with a slope of $29 \text{ mV}$ per 10-fold change in $[Ca^{++}]_o$ (or $[Mg^{++}]_o$). Our experiments (see Figs. 7 and 8) are in good agreement with this.

We can determine the value of $\sigma$ for PG membranes from the shift of the $g-V$ characteristic along the voltage axis in going from zero concentration of divalent cation to a finite value on the trans side. In 0.01 M KCl, going from 0 to 0.167 mM Mg$^{++}$ shifts the $g-V$ characteristic 47.5 mV (see Fig. 7). Substituting these values into equation 9, we obtain $\sigma = 1 \text{ charge per } 60 \text{ A}^2$, the value quoted at the beginning of section B part 1.

Now consider the effect of divalent cation addition to only the cis side. The reduction of $\Psi$ on this side will have three effects: (a) the interfacial concentration of K$^+$ on the cis side is reduced; this, of course, will not affect the $g-V$ characteristic very much. (b) The interfacial concentration of monazomycin is reduced. (c) An intramembrane potential difference is produced, with the cis side positive with respect to the trans side (Fig. 6 b). Effects (b) and (c) act in opposite directions. The reduction of interfacial monazomycin concentration lowers conductance at a given voltage, which formally shifts the $g-V$ characteristic to the right, whereas the intramembrane potential difference is now of the sign to shift the characteristic to the left. Moreover, these two effects are quantitatively comparable for the following reason: we can write for a PG membrane

$$g \propto [\text{monazomycin}]_o e^{\Psi_\sigma (\Psi_\sigma - \Psi_t)/kT} \tag{12}$$

where $[\text{monazomycin}]_o$ is the concentration of monazomycin at the interface and $\Psi_\sigma$ and $\Psi_t$ are the surface potentials on the cis and trans side, respectively. (In completely symmetrical solutions, $\Psi_\sigma = \Psi_t$ and equation 12 reduces to the

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2 In describing the effect of divalent cation added to the trans side, we neglected the reduction of interfacial $[K^+]$ both for simplicity and because this has a trivial effect on the $g-V$ characteristic compared with the effect of the intramembrane potential difference.
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Figure 7. The effect of divalent cation added to the side opposite to that on which monazomycin is present. The PG membrane was formed at room temperature in 0.01 M KCl. Monazomycin was then added to the front chamber, and after approximately 20 min, the g-V characteristic labeled \([\text{Mg}^{++}] = 0.0 \text{mM}\) was taken. MgSO\(_4\) was then added to the rear chamber to the concentrations indicated on the curves. It required only 1 min (the time to stir in the MgSO\(_4\)) for the new g-V characteristic to be established. Note the large shift of the g-V characteristic to the right along the voltage axis (see also Fig. 8). Similar results are obtained with Ca\(^{++}\). Slope of lines = e-fold conductance change per 4.0 mV; monazomycin concentration = 0.5 \(\mu\)g/ml; membrane area = 1 mm\(^2\).

Form of relation I). Combining with equation 10 (where \(i^+ = \text{monazomycin}^+\) and \(\Psi_o = \Psi_r\)) we have

\[
g \propto (e^\frac{nq(V-V_0)}{\varepsilon \kappa T})(e^\frac{q\Psi_v(n-s)}{\varepsilon \kappa T}).
\]  

(13)

If \(n \approx s\), as we have established, then conductance will essentially be invariant to changes in the surface potential on the cis side; that is, the g-V characteristic will not shift significantly upon addition of divalent cation to the same side on which monazomycin is present. This we observe experimentally in Fig. 9. (The small shift can be attributed either to small differences between \(n\) and \(s\), and/or to the decrease in current carrying ion (K\(^+\)) at the cis interface.)
In summary, addition of divalent cation to the trans side of PG membranes shifts the g-V characteristic to the right along the voltage axis because of the intramembrane potential difference. The field is of the sign to "drive" monazomycin out of the membrane. On the other hand, addition of divalent cation to the cis side hardly affects the g-V characteristic, because of the compensation of two large effects: (a) the internal field in the membrane, which is now of the sign to "drive" monazomycin into the membrane, and (b) the reduced monazomycin concentration at the interface, which makes it less available to be driven in by this field.

3. ASYMMETRIC UNI-UNIVALENT SALT CONCENTRATIONS Increasing the KCl concentration (in the absence of multivalent cations) on the trans side shifts the g-V characteristic of PG membranes to the right by over 50 mv for a 10-fold increase in KCl activity (Fig. 10). On the other hand, KCl gradients produce trivial shifts in the g-V characteristic of PE membranes (Fig. 7 b of Muller and Finkelstein, 1972). The reason for this difference is seen by comparing the theoretical potential profile for a PE membrane with that for a PG membrane in the presence of a KCl gradient (Fig. 11). In the former case, the diffusion EMF appears almost completely across the membrane, whereas in the latter case it appears virtually entirely in the aqueous solutions. To calculate the fraction of the EMF that appears in the aqueous solutions one must solve the Poisson-Boltzmann equation on the two sides of the membrane with the boundary conditions $\frac{d\Psi}{dx}|_{x=+} = \frac{d\Psi}{dx}|_{x=-}$ at each interface. It is then found (see, for example, Walz et al., 1969) that for the salt concentrations in our experiments, only a small fraction of the EMF appears in the aqueous solutions.
Figure 9. Comparison of the effect of divalent cation when added to the same side and to the opposite side on which monazomycin is present. The PG membrane was formed at room temperature in 0.01 M KCl. Monazomycin was then added to the front chamber, and, after approximately 15 min, curve A was obtained. MgSO₄ was then added to the front chamber to a concentration of 0.167 mM, and curve B was obtained 6 min later. Note the relatively small shift of the g-V characteristic compared with the large effect obtained when the same concentration of Mg²⁺ is added to the rear (Fig. 7). To confirm that this membrane does indeed respond properly to divalent cation added to the rear, MgSO₄ was then added to the rear compartment, and curve C was obtained 1 min later. (Similar effects are obtained with Ca²⁺.) Slope of lines A and B = e-fold conductance change per 4.6 mV; slope of line C = e-fold conductance change per 3.8 mV. (The difference in slope is not experimentally significant, and, in fact, a line of 4.6 mV can be reasonably fitted to the points in C.) Monazomycin concentration = 0.5 μg/ml; membrane area = 1 mm².
aqueous solution. Thus, in a PG membrane monazomycin does not "see" the EMF. Consequently, its conductance at \( V = \text{EMF} \) in a KCl gradient is the same as it was at \( V = 0 \) without the gradient; i.e., the \( g-V \) characteristic is shifted to the right along the voltage axis by the magnitude of the EMF.

In the preceding paper we demonstrated both theoretically and experimentally that a negative slope-conductance region could be developed in a PE membrane by the introduction of a positive EMF via a KCl gradient (see Fig. 7 of Muller and Finkelstein, 1972). This phenomenon depends on monazomycin being driven into the membrane by the EMF. With PG membranes, however, we have just shown that the diffusion EMF is not "seen" by monazomycin. Consequently, we cannot develop a negative slope-conductance region in a PG membrane by the addition of a KCl gradient in the absence of multivalent cations. If, however, significant concentrations of multivalent cations are present (either intentionally or through contamination), the surface potential will be more or less "clamped," and a significant fraction of the diffusion EMF produced by a KCl gradient will appear.

**Figure 10.** The effect of KCl added to the side opposite to that on which monazomycin is present. The PG membrane was formed at room temperature in a solution containing 10 mM KCl and 0.2 mM EDTA (to insure that polyvalent cation concentration is zero), with monazomycin in the front chamber. After approximately 25 min, curve A was obtained. KCl was then added to the rear chamber to a concentration of 43 mM, and curve B was obtained 1 min later. (As expected, an EMF of 31 mv [front positive] appeared upon the addition of KCl to the rear; this of course, was taken into account in calculating the conductance.) Note the large shift of the \( g-V \) characteristic to the right along the voltage axis, in contrast to the small shift of the characteristic to the left obtained with PE membranes (Fig. 7 b of Muller and Finkelstein, 1972). Slope of line A = \( e \)-fold conductance change per 5.1 mv; slope of line B = \( e \)-fold conductance change per 4.7 mv. Monazomycin concentration = 0.15 \( \mu \)g/ml; membrane area = 1 mm\(^2\).
Figure 11. Diagram comparing the potential profile for a PG and a PE membrane separating solutions with different KCl concentrations. The solid lines are the potential profiles for PG membranes, and the dashed lines are the profiles for PE membranes. In (A), no current is passed across the membrane; a potential difference of 60 mV exists across the membrane. (We are assuming, for simplicity, that the membrane is ideally cation selective.) Note that for the PE membrane this potential difference appears between the membrane interfaces and is therefore "seen" by monazomycin; for the PG membrane, there is no potential difference across the membrane proper. (If the membrane were not ideally selective, there would be a negative field within the membrane, even though there is a positive potential difference between the two solutions.) In (B), sufficient current is passed across the membrane to bring the potential difference between the two solutions to zero. Note that for the PE membrane there is now no potential difference between the two interfaces, whereas for the PG membrane there is a potential difference of -60 mV. (This is independent of the degree of cation selectivity; it is only dependent on the difference in surface potentials at the two interfaces.) These diagrams should make clear the basis for the shift of the $g-V$ characteristic to the right in Fig. 10.

across the membrane proper and be "seen" by the monazomycin. Under these circumstances the rectifying $I-V$ characteristic of a PG membrane is converted to one with a negative slope region.

Addition of KCl to the cis side produces two compensating effects: (a) the reduction of monazomycin concentration at the interface, which formally shifts the $g-V$ characteristic to the right, and (b) the production of a negative

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12 It was for this reason that Mg$^{2+}$ was present in our flip-flop experiment (Fig. 10 of Muller and Finkelstein, 1972).
diffusion EMF (which is measured by our electrodes but does not appear between the two membrane interfaces) that shifts the \( g-V \) characteristic to the left. As with the \( cis \) addition of divalent cation, if \( s \) and \( n \) are approximately equal, the two effects cancel and there is no shift in the \( g-V \) characteristic. This we see experimentally in Fig. 12.

4. SUMMARY OF THE EFFECT OF LIPID CHARGE. Our over-all motif has been that the action of monazomycin on PE and PG membranes is *intrinsi*...
cally the same. Apparent differences in behavior are attributable to differences in the boundary conditions at the membrane interfaces, arising from the (near) absence of surface charge on PE membranes and the existence of a large negative surface charge on PG membranes. Furthermore, the effect of such charge is quantitatively calculable from simple diffuse double layer theory.

DISCUSSION

While developing the theory, we included considerable discussion and interpretation of the effect of lipid charge. In this section we shall make some additional comments on both the applicability of diffuse double layer theory to charged bilayers and the relevance of the effects obtained in this system to those obtained in biological membranes.

A. Diffuse Double Layer Theory Applied to Charged Bilayers

1. ASSUMPTIONS OF THE THEORY It is far beyond the scope of this paper to present a critique of the explicit and implicit assumptions underlying diffuse double layer theory. There are complexities at all levels of analysis. It is even difficult to justify the correctness of the Poisson-Boltzmann equation, the cornerstone of the theory. (See Rutgers [1954] for a discussion of this problem.) There are, however, two specific assumptions that we wish to emphasize: one is that the discrete negative charges of the phosphate groups are smeared out to give a uniform surface charge density, \( \sigma \). The other is that the ions (which are considered as point charges averaged out to a volume density, \( \rho \)) in solution interact with surface charge in a purely electrostatic fashion. In particular, specific absorption or binding of ions from solution with the surface charge groups is excluded. Neither is the size of the ion a consideration; the only relevant property of an ion is its valence. The effects we have reported of divalent cations at concentrations \( \frac{1}{100} \)th those of univalent ions are quantitatively consistent with this simple theory and do not require any assumptions about binding of Ca\(^{++}\) or Mg\(^{++}\) to the surface.

2. COMPARISON OF MONAZOMYCIN RESULTS TO THOSE OBTAINED WITH K\(^{+}\) CARRIERS This absence of binding has been emphasized by McLaughlin et al. (1970, 1971) in their analysis of the effect of divalent cations on the permeability induced in PG membranes by K\(^{+}\) carriers such as nonactin. To be sure, there are small quantitative differences between Ca\(^{++}\) and Mg\(^{++}\) (comparable effects are obtained with Ca\(^{++}\) at concentrations about half those required with Mg\(^{++}\)), indicating some specific ion binding. Nevertheless, these authors properly stress that the most important factor operative in the action of Ca\(^{++}\) and Mg\(^{++}\) on negatively charged lipid bilayers is simple electrostatic interaction.

The agreement between the divalent cation effects obtained by McLaughlin
et al. using the K⁺ carrier nonactin and our results using monazomycin is very gratifying. (In fact, their results prompted our own investigation of the effect of lipid charge.) They determined \( \sigma \) of cholesterol-free PG membranes to be 1 charge per 38 \( A^2 \), in very good agreement with our result on PG membranes with cholesterol of 1 charge per 60 \( A^2 \). (We cannot say whether this difference results from dilution of the surface charge by cholesterol or whether it merely reflects uncertainties in the experimental determination of \( \sigma \).)

The over-all agreement of our results with those of McLaughlin et al. lends strong support to the underlying theory, since the nonactin and monazomycin systems are so different. Nonactin forms a 1:1 complex with K⁺ and acts as a simple carrier of that ion; conductance is linear with nonactin concentration and not voltage-dependent. Monazomycin, on the other hand, however it may act, certainly does not function as a simple carrier. Conductance is proportional to a large power of the antibiotic concentration and is strongly voltage dependent.

Because of these differences, the effects of Ca⁺⁺ and Mg⁺⁺ on monazomycin-treated membranes are much more “spectacular,” even though their mechanism of action is the same in both systems. Symmetrical addition of Ca⁺⁺ that lowers \( \Psi \) by 60 mv reduces the conductance of a nonactin-treated membrane only by a factor of 10 (by reducing interfacial [K⁺] by this amount), whereas it reduces the conductance of a monazomycin-treated membrane by at least a factor of \( 10^5 \). (In fact, we generally neglected the effect of divalent cations on interfacial [K⁺], since in the monazomycin system the dependence of conductance on [K⁺] is “merely” linear; in the nonactin-treated membrane, this linear dependence is the only one operative.) Furthermore, the large trans effect of Ca⁺⁺ and Mg⁺⁺ in monazomycin-treated PG membranes hardly exists in nonactin-treated membranes, since the latter are not voltage dependent. In short, there is tremendous amplification of divalent cation action with monazomycin, because conductance depends on a large power of the antibiotic concentration and is exquisitely sensitive to voltage.

3. EFFECT OF LOCAL ANESTHETICS We do not wish to leave the impression that the \( g-V \) characteristic cannot be shifted by genuine binding of positive charges to the membrane. In fact we see precisely this with positively charged local anesthetics such as procaine and tetracaine (Fig. 13). (Interestingly, tetracaine is effective on bilayers at much lower concentrations than procaine, a result consistent with their potency as local anesthetics.) The small concentrations at which tetracaine (a univalent cation) is effective preclude simple electrostatic interaction as a mechanism and demand genuine binding of the anesthetic to the membrane.

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13 In their 1970 paper, this was given as 1 charge per 50 \( A^2 \) (McLaughlin et al., 1970).
B. Relevance to Biological Membranes

It is most unlikely that shifts in the $g-V$ characteristic of monazomycin-treated PG membranes that occur upon symmetrical alterations of KCl (Fig. 2) or of Mg++ and Ca++ (Fig. 3) are relevant to biological excitable membranes. These effects depend upon the dynamic equilibrium of monazomycin between membrane and solution. It is almost certain that the molecules responsible for the voltage-dependent conductance elements in excitable cells are more or less permanently associated with the membrane (at least on the time scales we have been considering) and are not rapidly exchanging with molecules in the cytoplasm.

On the other hand, the trans effect with Mg++ and Ca++ in PG membranes (Figs. 7 and 8) is directly relevant to the shifts in the characteristics of the sodium and potassium channels observed in axons upon changing the concentration of these ions in the external medium (Frankenhaeuser and Hodgkin, 1957; Gilbert and Ehrenstein, 1969). In fact the explanation given for these shifts is in essence the same as we have invoked for the monazomycin system, namely that Mg++ and Ca++ alter the surface potential at the external surface and thereby create an internal field within the membrane that is "seen" by the voltage-dependent element. The magnitudes of the effects seen biologically (about a 20–25 mv shift in the characteristics for a 10-fold change in divalent ion concentration) are comparable to those for the monazomycin system. This agreement cannot be used, however, to implicate monazomycin, or a molecule similar to it, in the voltage-dependent conductance elements of biological membranes. If there is a voltage-dependent element in a membrane that bears a negative surface charge, the effects we have discussed should be observed, independent of the molecular subtleties underlying the voltage-dependent phenomenon.

One difference between the model membrane and the biological membrane should be noted. In the bilayer membrane it is reasonable to assume that $\sigma$ (and hence $\Psi_0$) is uniform over the entire surface (particularly if the membrane is formed from a single lipid). On the other hand, there is no reason a priori to believe that this is so in plasma membranes; in particular we cannot assume that the density of negatively charged groups in the vicinity of the potassium and sodium channels is the same. Probably for this reason the voltage shifts of the potassium and sodium conductances differ somewhat.

The shifts in the $g-V$ characteristic produced by changes in KCl concentra-

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14 Most authors have attributed the alteration of surface potential produced by Mg++ and Ca++ to "binding" of these ions to negative surface-charge groups. As we have discussed previously, the major effect of divalent cations on monazomycin-treated PG membranes is predicted from diffuse double layer theory without invoking binding. The biological data are equally well handled with this theory. (See McLaughlin et al., 1971, for further discussion of this point.)
tion on the trans side of monazomycin-treated PG membranes (Fig. 10) are completely analogous to the shifts of the sodium conductance produced by changes of uni-univalent salt concentration on the inside of the squid axon (Chandler et al., 1965). The interpretation and analysis given by these authors is identical to the one we have presented; namely, they assume a negative fixed charge density exists on the inner surface of the membrane and that changes in salt concentration alter the surface potential and thus create an internal field within the membrane which is "seen" by the voltage-dependent system in the membrane but is not measured by the recording electrodes.

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