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Interaction of Gentamicin and Spermine with Bilayer Membranes Containing Negatively Charged Phospholipids†

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ABSTRACT: We measured the electrophoretic mobility of multilamellar phospholipid vesicles, the ^{31}P NMR spectra of both sonicated and multilamellar vesicles, and the conductance of planar bilayer membranes to study the binding of spermine and gentamicin to membranes. Spermine and gentamicin do not bind significantly to the zwitterionic lipid phosphatidylcholine. We measured the concentrations of gentamicin and spermine that reverse the charge on vesicles formed from a mixture of phosphatidylcholine and either phosphatidylserine or phosphatidylinositol. From these measurements, we determined that the intrinsic association constants of the cations with these negative lipids are all about 10 M^{-1}. This value is orders of magnitude lower than the apparent binding constants reported in the literature by other groups because the negative electrostatic surface potential of the membranes and the resultant accumulation of these cations in the aqueous diffuse double layer adjacent to the membranes have not been explicitly considered in previous studies. Our main conclusion is that the Gouy-Chapman-Stern theory of the aqueous diffuse double layer can describe surprisingly well the interaction of gentamicin and spermine with bilayer membranes formed in a 0.1 M NaCl solution if the negative phospholipids constitute <50% of the membrane. Thus, the theory should be useful for describing the interactions of these cations with the bilayer component of biological membranes, which typically contain <50% negative lipids. For example, our results support the suggestion of Sastrasinh et al. [Sastrasinh, M., Krauss, T. C., Weinberg, J. M., & Humes, H. D. (1982) *J. Pharmacol. Exp. Ther. 222*, 350-358] that phosphatidylinositol is the major binding site for gentamicin in renal brush border membranes.

The interaction of gentamicin and spermine with membranes is of interest for several reasons. Gentamicin is an aminoglycoside antibiotic used to treat infections caused by Gram-negative bacteria; it interferes with protein synthesis in susceptible microorganisms. The clinical utility of gentamicin and the related aminoglycoside antibiotics is limited by their nephrotoxic and ototoxic effects [e.g., see Sande & Mandell (1980)], and several investigators have suggested that these toxic effects are related to the interaction of the antibiotics with negatively charged phospholipids in biological membranes (Sastrasinh et al., 1982; Laurent et al., 1982; Brasseur et al., 1984). Spermine is produced within cells and may affect a number of different membrane functions [e.g., see Ballas et al. (1983), Schuber et al. (1983), Hong et al. (1983), and Koenig et al. (1983a,b)].

The molecular structures of gentamicin (Cooper et al., 1971; Daniels, 1975) and spermine are illustrated in Figure 1: both spermine and gentamicin have about 3.5 positive charges at pH 7.4 (Josepovitz et al., 1982). Several groups have studied

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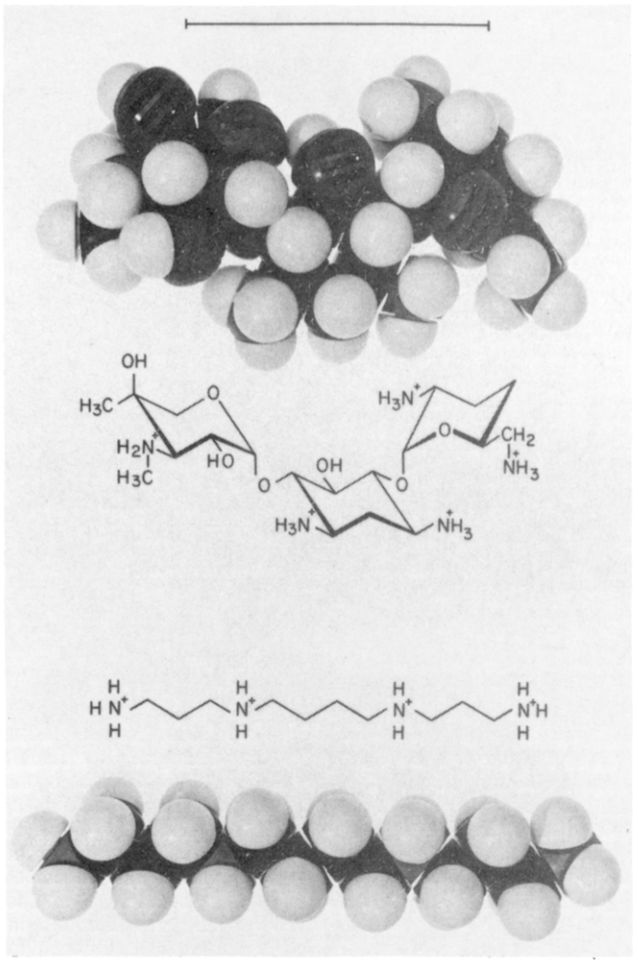

FIGURE 1: Molecular formulas and CPK models of gentamicin C_{1a} (top) and spermine (bottom). Gentamicin has a maximum of five and spermine a maximum of four positive charges. The bar corresponds to 10 Å.

the binding of gentamicin to biological membranes (Lodhi et al., 1976; Just & Habermann, 1977; Sastrasinh et al., 1982), to phospholipid monolayers (Auslander et al., 1975; Lodhi et al., 1980; Lullmann & Vollmer, 1982), and to phospholipid bilayers (Alexander et al., 1979; Laurent et al., 1982; Brasseur et al., 1984). The fixed negative charges on these membranes produce a negative electrostatic potential in the aqueous diffuse double layer adjacent to the surface (McLaughlin, 1977). For example, the surface potential of a bilayer membrane formed from the negative lipid phosphatidylserine (PS)[1] or phosphatidylglycerol (PG) is about −80 mV in a 0.1 M NaCl solution (Eisenberg et al., 1979; Alvarez et al., 1983; McLaughlin et al., 1983). The surface potential of a bilayer membrane that contains 20% negative phospholipids, a value characteristic of many biological membranes, is about −40 mV in 0.1 M NaCl (McLaughlin et al., 1981). According to the Boltzmann relation, the concentration of a tetravalent cation in the aqueous phase at the surface of these membranes will be orders of magnitude larger than the concentration in the bulk aqueous phase, and the apparent association constant, K_A, will be orders of magnitude larger than the intrinsic association constant, K_I. K_A is related to K_I by the Boltzmann expression:

$$K_A = K_I \exp[-zF\psi(0)/(RT)] \simeq K_I 10^{-z\psi(0)/60} \quad (1)$$

where F is the Faraday constant, R is the gas constant, T is the absolute temperature, z is the valence of the cation, which is assumed to be a point charge, and $\psi(0)$ is the electrostatic potential (in millivolts) in the aqueous phase at the surface of the membrane. Any factor that affects $\psi(0)$ will affect K_A and the binding of gentamicin to the membrane.

In the past, the binding of gentamicin to membranes has been described by double-reciprocal and Scatchard plots [e.g., see Just & Habermann (1977), Lodhi et al. (1980), and Sastrasinh et al. (1982)]. This is equivalent to assuming that gentamicin forms only 1:1 complexes with the binding sites (Langmuir adsorption isotherm) and that electrostatic potentials can be ignored. We suggest that a Langmuir adsorption isotherm appears to describe the binding of gentamicin to membranes containing negative lipids for the following reason. As the concentration of gentamicin in the bulk aqueous phase increases, more gentamicin binds to the membrane. The surface charge and surface potential of the membrane become less negative, the concentration of gentamicin in the aqueous phase adjacent to the membrane does not increase proportionally with the bulk aqueous concentration, and the binding appears to saturate. This apparent saturation is due mainly to an electrostatic effect, a decrease in the magnitude of $\psi(0)$ in eq 1, and not to a saturation in the number of binding sites (McLaughlin & Harary, 1976; McLaughlin, 1977).

The simplest way to account for the effect of electrostatic potentials on the adsorption of gentamicin and spermine to membranes is to follow Stern (1924) and combine the theory of the aqueous diffuse double layer, due originally to Gouy (1910) and Chapman (1913), with an adsorption isotherm (e.g., Langmuir). The Gouy–Chapman–Stern theory can explain three observations in the literature related to the binding of gentamicin to membranes. First, Laurent et al. (1982) and Brasseur et al. (1984) observed that the number of moles of gentamicin bound to bilayer membranes containing both zwitterionic lipids and the negative lipid phosphatidylinositol (PI) increased in a superlinear manner with the PI content ($0 < PI < 27\%$). They concluded that the binding of gentamicin to PI was "cooperative in nature". Our ζ potential measurements confirm their observations, but the Gouy–Chapman–Stern theory predicts that the binding of gentamicin to PI is *not* cooperative when PI comprises <50% of the membrane. The theory predicts, and experiments confirm, that an increase in the mole fraction of negative lipids in the membrane increases the magnitude of the negative electrostatic potential adjacent to the membrane [see Figure 2 of McLaughlin (1977)]. Equation 1 illustrates that an increase in the magnitude of $\psi(0)$ increases the apparent binding constant of gentamicin to the membrane. This effect can account, at least qualitatively, for the apparent cooperativity.

Second, Laurent et al. (1982) and Brasseur et al. (1984) observed that the binding of gentamicin to liposomes containing negative lipids increases as the NaCl concentration decreases. Equation 1 can account for this observation because the surface potential adjacent to a bilayer membrane containing anionic phospholipids becomes more negative when the ionic strength decreases, as predicted theoretically [see Figure 3 of McLaughlin (1977)].

Third, Alexander et al. (1979) and Lullmann & Vollmer (1982) observed that the binding of gentamicin to bilayer membranes containing negative lipids decreases when the concentration of calcium increases. This effect can also be

[1] Abbreviations: DMPS, dimyristoylphosphatidylserine; G, gentamicin; G_{M1}, monosialoganglioside; G_{D1a}, disialoganglioside; G_{T1b}, trisialoganglioside; MOPS, 3-(*N*-morpholino)propanesulfonic acid; PC, phosphatidylcholine; PI, phosphatidylinositol; PS, phosphatidylserine; S, spermine; PG, phosphatidylglycerol; FCCP, carbonyl cyanide *p*-(trifluoromethyl)phenylhydrazone; EDTA, ethylenediaminetetraacetic acid.

accounted for by eq 1 because increasing the concentration of calcium decreases the magnitude of the potential adjacent to a membrane containing negative phospholipids. This effect can be described by the Gouy–Chapman–Stern theory (McLaughlin et al., 1981; Alvarez et al., 1983).

We performed the experiments described in this report to test the ability of the Gouy–Chapman–Stern theory to describe the adsorption of gentamicin and spermine to charged membranes. The theory makes a number of specific predictions. For a membrane with a high density of negative surface charges, the slopes of the surface or ζ potential vs. $\log_{10}$ [cation] curves should be approximately $2.3RT/(zF)$, where z is the valence of the cation in the aqueous phase. Thus, for monovalent, divalent, and tetravalent cations, the slopes should be about 58, 29, and 14 mV/decade of cation concentration. These predictions have been confirmed experimentally for monovalent and divalent cations [e.g., see McLaughlin (1977, 1983)]; here, we confirm the predictions for spermine and gentamicin.

Theory

The Gouy–Chapman theory of the diffuse double layer has been reviewed many times in the past (Grahame, 1947; Verwey & Overbeek, 1948; Haydon, 1964; Mohilner, 1966; Sanfeld, 1968; Barlow, 1970; Bockris & Reddy, 1970; Sparnaay, 1972; Aveyard & Haydon, 1973; McLaughlin, 1977, 1983). An equation derived by Grahame (1947) relates the surface charge density, σ, and the electrostatic potential in the aqueous phase at the surface, $\psi(0)$:

$$\sigma = \pm\{2\epsilon_r\epsilon_0 RT\sum_i c_i\,[\exp[-z_iF\psi(0)/(RT)] - 1]\}^{1/2} \quad (2)$$

where ϵ_r is the dielectric constant of the aqueous solution, ϵ_0 is the permittivity of free space, and c_i is the concentration of ions of valence z_i in the bulk aqueous solution.

The surface charge density in eq 2 is the sum of the surface concentrations of all charged species. We assume, for mathematical simplicity, that both spermine and gentamicin molecules have four positive charges in the bulk aqueous phase and in the aqueous diffuse double layer. (The assumption is not strictly correct because the charge on these molecules depends on the pH and the pH at the membrane–solution interface is a function of the surface potential.) We also assume that cations do not adsorb to the zwitterionic lipid PC, that anions do not adsorb to the membrane, and that the binding of monovalent and tetravalent cations to the negative lipids is competitive. When the membrane consists of a mixture of the monovalent anionic lipid phosphatidylserine (PS) and the zwitterionic lipid phosphatidylcholine (PC) and the aqueous phase contains spermine (S), the surface charge density is

$$\sigma = (-\{PS\} + 3\{S\text{–}PS\} + 2\{S\text{–}(PS)_2\})e \quad (3)$$

where {PS} is the surface concentration of PS, {S–PS} is the surface concentration of complexes formed between one S molecule and one PS molecule, $\{S\text{–}(PS)_2\}$ is the surface concentration of complexes formed between one S molecule and two PS molecules, and e is the magnitude of the electronic charge. The extension to gentamicin, where we also assume that the tetravalent cation can form complexes with three and four PS molecules, is apparent.

We use Langmuir adsorption isotherms (mass action) to relate the surface concentrations in eq 3 to the aqueous concentrations of spermine, $[S]_{x=0}$, and sodium, $[Na]_{x=0}$, at the membrane–solution interface, which is located at $x = 0$. For membranes containing PS, the surface concentrations of the complexes are

$$\{Na\text{–}PS\} = K_{Na\text{–}PS}\{PS\}[Na]_{x=0} \quad (4)$$

$$\{S\text{–}PS\} = K_{S\text{–}PS}\{PS\}[S]_{x=0} \quad (5)$$

$$\{S\text{–}(PS)_2\} = K_{S\text{–}(PS)_2}\{PS\}^2[S]_{x=0} \quad (6)$$

The extension to gentamicin, where we also consider 1:3 and 1:4 cation–lipid complexes with PS and PI, is obvious. Cohen & Cohen (1981) may be consulted for a lucid discussion of more complex adsorption isotherms.

We use the Boltzmann equation to relate the concentrations in the aqueous phase at the surface of the membrane to the concentrations in the bulk aqueous phase:

$$[Na]_{x=0} = [Na]\exp[-F\psi(0)/(RT)] \quad (7)$$

$$[S]_{x=0} = [S]\exp[-4F\psi(0)/(RT)] \quad (8)$$

Finally, we note that the total surface concentration of PS, $\{PS\}^{tot}$, is given by

$$\{PS\}^{tot} = \{PS\} + \{Na\text{–}PS\} + \{S\text{–}PS\} + 2\{S\text{–}(PS)_2\} \quad (9)$$

We solve eq 2–9 numerically to obtain the predicted dependence of $\psi(0)$ on the aqueous concentration of spermine and gentamicin.

The ζ potential is the electrostatic potential at the hydrodynamic plane of shear. Eisenberg et al. (1979), Alvarez et al. (1983), and Rooney et al. (1983) have presented evidence that the plane of shear is located 2 Å from the surface of a phospholipid bilayer membrane in a solution containing 0.1 M NaCl. We obtained the theoretically predicted values of the ζ potential from the surface potential by using the appropriate Gouy–Chapman expression for the potential profile in the aqueous phase (see the Appendix).

Materials and Methods

Bovine brain phosphatidylserine (PS), dimyristoylphosphatidylserine (DMPS), bovine liver phosphatidylinositol (PI), egg phosphatidylcholine, and diphytanoylphosphatidylcholine (PC) were purchased from Avanti Polar Lipids, Inc. (Birmingham, AL); 3-(*N*-morpholino)propanesulfonic acid (MOPS) was from P-L Biochemicals, Inc. (Milwaukee, WI); spermine, 98% pure, was from Aldrich Chemical Co. (Milwaukee, WI). Monosialoganglioside (G_{M1}), disialoganglioside (G_{D1a}), and trisialoganglioside (G_{T1}) were purchased from Supelco (Bellefonte, PA). Gentamicin sulfate, which consists of a mixture of gentamicin C_1, C_{1a}, and C_2, was obtained from the Schering Corp. (Bloomfield, NJ). Water was deionized in a Super-Q system (Millipore Corp., Bedford, MA), double distilled in a quartz still, and stored in quartz flasks. The solutions for the electrophoresis experiments contained 0.1 M NaCl buffered to pH 7.4 with 1 mM MOPS for the gentamicin experiments and 0.1 M NaCl buffered to pH 7.0 with 10 mM MOPS for the spermine experiments. The pH of the solution containing the vesicles and either spermine or gentamicin was adjusted, if necessary, before mobility measurements were made.

Multilamellar vesicles for the microelectrophoresis measurements were prepared according to the procedure described by Bangham et al. (1974). All electrophoretic mobility measurements were performed at 25 °C using a Rank Bros. Mark I microelectrophoresis apparatus (Bottisham, Cambridge, U.K.). Measurements were made at the stationary layer (Henry, 1938), and the current was monitored to check that electrode polarization did not occur. The electrophoresis tube was cleaned regularly with chromic acid to prevent buildup of lipid deposits that change the position of the stationary layer. When the concentrations of either spermine or

gentamicin were low, it was necessary to first equilibrate the electrophoresis tube with the solution containing the tetravalent cation and to use very low concentrations of vesicles to prevent a significant loss of the cation from the aqueous phase.

The ζ potential, ζ, was calculated from the electrophoretic mobility, u, by using the Helmholtz–Smoluchowski equation:

$$\zeta = \eta u/(\epsilon_r \epsilon_0) \quad (10)$$

where η is the viscosity of the solution, ϵ_r is the dielectric constant of the solution, and ϵ_0 is the permittivity of free space.

We used two methods to measure the binding of gentamicin to sonicated brain PS vesicles. First, we used ^{31}P NMR (see below). Second, we used the mobility of multilamellar vesicles as an indirect measure of the free concentration of gentamicin in a solution containing sonicated vesicles. Sonicated vesicles for the latter experiments were prepared as described by Barenholz et al. (1977) in a solution containing 0.1 M NaCl and 10 mM MOPS, pH 7.4. A phosphate assay (Lowry & Tinsley, 1974) was used to estimate the final concentration of lipid in the sonicated vesicle preparation.

Planar bilayers were formed from a 1% solution of 1:1 bovine brain PS:diphytanoyl-PC in decane. The lipid solution was applied to a 1.6-mm aperture in a Teflon chamber. The aqueous phase contained 0.1 M KCl buffered to pH 7.5 with 1 mM MOPS at room temperature (20–22 °C). The steady-state conductance was determined by applying a voltage of 25 mV or less to the membranes after equilibration with either 0.5 μM aqueous nonactin or FCCP. The conductance was measured again after addition of stock solutions containing gentamicin or spermine. The gentamicin or spermine stock solutions were buffered to pH 7.5, and the pH in the bilayer chamber was checked before and after the addition of the cations. The electrostatic potential within the bilayer is defined with respect to the potential within a PC bilayer and is calculated from (McLaughlin, 1977; Benz & McLaughlin, 1983)

$$G/G_0 = \exp[\pm F\Delta\psi/(RT)] \quad (11)$$

where G is either the nonactin or the FCCP conductance of the bilayer formed from a 1:1 PC:PS mixture, G_0 is the nonactin or FCCP conductance of a pure PC bilayer, and $\Delta\psi$ is the difference between the electrostatic potential within the two bilayer membranes. The plus sign is used for FCCP conductance, and the minus sign is used for nonactin conductance.

We estimated the K–PS intrinsic association constant by measuring the ζ potential (-54.8 ± 1.0 mV, $n = 40$) of 1:1 PC:PS multilamellar vesicles in 0.1 M KCl solutions. A ζ potential of −55 mV corresponds to a surface potential of −71 mV if the plane of shear is 2 Å from the bilayer surface (Eisenberg et al., 1979; Alvarez et al., 1983). We calculated an intrinsic K–PS association constant of about 0.5 M^{-1} from the surface potential of −71 mV using the Gouy–Chapman–Stern theory [e.g., see Eisenberg et al. (1979)].

^{31}P NMR spectra of sonicated phosphatidylserine vesicles were obtained at 145.7 MHz on a Bruker WH 360 spectrometer. Sonicated PS vesicles were prepared in 0.1 M sodium chloride and 5 mM MOPS, pH 7.4; the method of preparation is described elsewhere (McLaughlin et al., 1981). Each data point was determined from a fresh sample of vesicles. Unless otherwise noted, 50 mg of PS was used for each experiment. All experiments were performed at 25 °C.

Low concentrations of manganese broaden the ^{31}P NMR line width of sonicated phospholipid vesicles. The broadening is proportional to the free concentration of manganese in the aqueous phase adjacent to the phosphate group, and this concentration is proportional to the Boltzmann factor $\exp[-2F\psi_p/(RT)]$, where ψ_p is the electrostatic potential at the phosphodiester group. Changes in the width of the ^{31}P NMR signal in the presence of manganese can be used to calculate changes in the potential at the phosphodiester group upon addition of gentamicin. The free manganese concentration in the bulk aqueous medium (0.2 μM) was established by passing the sonicated vesicle suspension through a Sephadex G-200 column equilibrated with 0.2 μM manganese chloride and known concentrations of gentamicin. Under these conditions, the ^{31}P NMR signal from PS molecules in the outer monolayer is substantially broadened, but the ^{31}P NMR signal from PS molecules on the inner monolayer is unaffected. The line width of the broad signal from the outer PS molecules was measured by using the π, τ, $\pi/2$ radio-frequency pulse sequences with a value of τ that nulled the narrow signal from the inner PS molecules (Lau et al., 1981). The effect of manganese on the transverse relaxation rate, $1/T_{2p}$, was calculated from the relationship $1/T_{2p} = \pi\Delta\nu_p$, where $\Delta\nu_p$ is the increase in the line width of the ^{31}P NMR signal upon addition of manganese.

If we assume that the number of manganese binding sites on the PS vesicle remains constant upon addition of gentamicin, the ratio of the ^{31}P NMR line width in the presence, $1/T_{2p}$, and in the absence, $1/T_{2p}^0$, of gentamicin is given by

$$(1/T_{2p})/(1/T_{2p}^0) = \exp[-2F\Delta\psi_p/(RT)] \quad (12)$$

where $\Delta\psi_p$ is the change in the potential at the phosphodiester group upon the addition of gentamicin (Lau et al., 1981). If we consider the effect of gentamicin on the number of available manganese binding sites, a more complicated expression results (McLaughlin et al., 1983).

We added gentamicin to the PS vesicle samples before passing them through a Sephadex G-200 column equilibrated with gentamicin and manganese. Control experiments showed that the free concentration of gentamicin in the sample was greater than the gentamicin concentration in the column. Under these conditions, the columns sequestered the excess gentamicin in the sample.

For the ^{31}P NMR measurements on multilamellar PS vesicles, 50 mg of PS was dried under a stream of nitrogen, and residual solvent was removed overnight under a high vacuum. The sample was dispersed in 4 mL of 0.1 M NaCl and 5 mM MOPS, pH 7.4. The final pH (7.4) and also the gentamicin and magnesium concentrations were adjusted by using a freeze–thaw procedure to equilibrate the internal volume of the liposomes with the external aqueous medium (Hope & Cullis, 1980).

Results

ζ Potential Measurements. (A) Spermine. Figure 2A illustrates the effect of spermine on the ζ potential of multilamellar vesicles formed from either the zwitterionic lipid egg PC, the negative lipid PS, or 1:1 PC:PS mixtures. Spermine has no effect on the ζ potential of the PC vesicles, indicating that it does not adsorb significantly to this zwitterionic lipid at the concentrations we have used. On the other hand, spermine has a large effect on the ζ potentials of PS and 1:1 PC:PS vesicles. High concentrations of spermine cause the ζ potential to reverse sign and become positive.

Figure 2B illustrates the effect of spermine on the ζ potentials of 5:1, 10:1, and 20:1 PC:PS vesicles. About 0.03 M spermine reduces the ζ potentials of each of these vesicles to zero. If we assume that spermine and phosphatidylserine form only 1:1 S–PS complexes, we can easily calculate the intrinsic 1:1 binding constant, $K_{S\text{–}PS}$, from the aqueous concentration of spermine required to cause charge reversal, $[S]^{rev}$, which

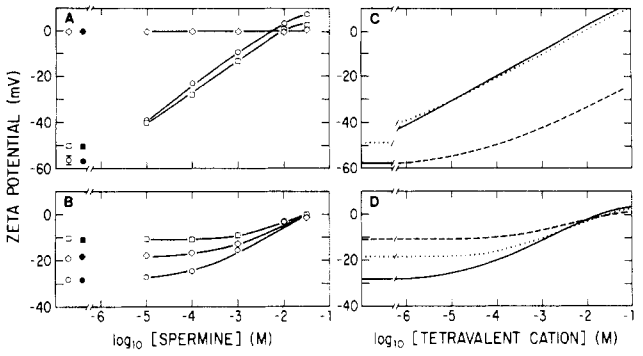

FIGURE 2: Effect of the concentration of the tetravalent cation spermine on the ζ potentials of multilamellar vesicles. The solutions contained the indicated concentration of spermine and 0.1 M NaCl and were buffered with 10 mM MOPS to pH 7.0. The filled symbols indicate the effect of 100 μM EDTA. (A) Experimental results obtained with egg phosphatidylcholine vesicles (PC) (diamonds), brain phosphatidylserine vesicles (PS) (circles), and 1:1 PC:PS vesicles (squares). The standard deviations of the 20 measurements were typically about 2 mV (e.g., lower left point). The curves in (A) and (B) were drawn only to guide the eye; they have no theoretical significance. (B) Experimental results obtained with 5:1 PC:PS vesicles (circles), 10:1 PC:PS vesicles (diamonds), and 20:1 PC:PS vesicles (squares). (C) Theoretical predictions of the Gouy–Chapman–Stern theory: PS vesicles (solid curve); 1:1 PC:PS vesicles (dotted curve). The dashed curve was calculated from the theory by assuming that spermine, S, does not adsorb to PS. The solid and dotted curves were both calculated by using an S–PS intrinsic association constant of $K_{S\text{-}PS} = 10\ M^{-1}$ (see eq 5) and an S–$(PS)_2$ intrinsic association constant of $K_{S\text{-}(PS)_2} = 1000\ M^{-1}\ Å^2$ (see eq 6). (D) Theoretical predictions of the Gouy–Chapman–Stern theory: 5:1 PC:PS vesicles (solid line); 10:1 PC:PS vesicles (dotted line); 20:1 PC:PS vesicles (dashed line). The intrinsic association constants are the same as in (C).

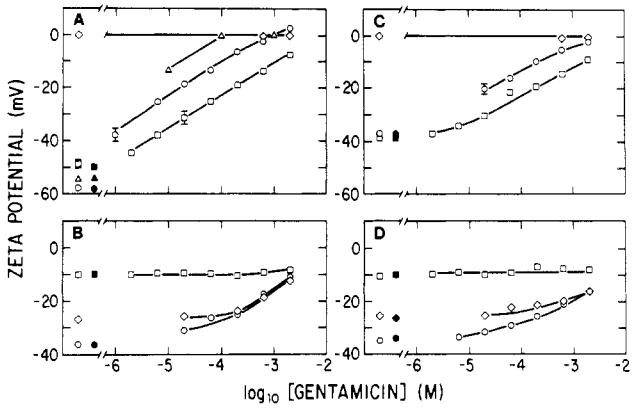

FIGURE 3: Effect of gentamicin concentration on the ζ potentials of PC, PS, dimyristoylphosphatidylserine (DMPS), phosphatidylinositol (PI), PC:PS, and PC:PI vesicles. The solutions contained 0.1 M NaCl and were buffered with 1 mM MOPS to pH 7.4. The filled symbols indicate the effect of 100 μM EDTA. The curves are drawn only to guide the eye; they have no theoretical significance. (A) PC vesicles (diamonds); 1:1 PC:PS vesicles (squares); PS vesicles (circles); DMPS vesicles (triangles). (B) 2.5:1 PC:PS vesicles (circles); 5:1 PC:PS vesicles (diamonds); 20:1 PC:PS vesicles (squares). (C) PC vesicles (diamonds); PI vesicles (circles); 1:1 PC:PI vesicles (squares). (D) 2.5:1 PC:PI vesicles (circles); 5:1 PC:PI vesicles (diamonds); 20:1 PC:PI vesicles (squares).

is 0.03 M. When the ζ potential is zero, the charge density is zero, and eq 3 illustrates that the surface concentration of negatively charged PS molecules, {PS}, is 3 times the surface concentration of triply positive S–PS complexes, {S–PS}. Furthermore, when the charge density and surface potential are zero, eq 8 illustrates that the aqueous concentration of spermine at the membrane–solution interface equals the bulk concentration: $[S]_{x=0} \simeq [S]$. It follows from eq 3 and 5 that $K_{S\text{-}PS} = 1/(3[S]^{rev}) = 10\ M^{-1}$. Note in Figure 2B that the value of $[S]^{rev}$ does not depend significantly on {PS} when {PS} is low. This result is consistent with our postulate that spermine forms mainly 1:1 complexes with PS when {PS} is low. When the vesicles are enriched in PS, the value of $[S]^{rev}$ shifts to lower values. For 1:1 PC:PS vesicles, the value of $[S]^{rev} = 0.01$ M, whereas for PS vesicles $[S]^{rev} = 0.005$ M (see Figure 2A). This shift can be explained by postulating that spermine forms both higher order and 1:1 complexes with PS, as illustrated by the theoretical curves in Figure 2C,D.

The dashed theoretical curve in Figure 2C illustrates the predicted effect of a tetravalent cation on the ζ potential of a PS vesicle, assuming the cation does not adsorb to the membrane. Spermine (Figure 2A, circles) has a much larger effect on the ζ potential than predicted by this "screening" curve; thus, we must invoke some specific adsorption of spermine to PS to account for the data. The other theoretical curves in Figure 2C,D illustrate the predictions of the Gouy–Chapman–Stern theory assuming that $K_{S\text{-}PS}$ equals 10 M^{-1}, the association constant required to explain the charge reversal data obtained when {PS} is low (see Figure 2B), and that $K_{S\text{-}(PS)_2}$ (eq 6) equals $10^3\ M^{-1}\ Å^2$, an association constant that accounts for the decrease in the concentration of spermine required to produce charge reversal as {PS} increases (Figure 2A). (If {PS} = 1/70 $Å^2$, this value of $K_{S\text{-}(PS)_2}$ corresponds to a value of $K_{S\text{-}(PS)_2}\{PS\} = 14\ M^{-1}$.) Note that the simple Gouy–Chapman–Stern theory can explain all the qualitative features of the data.

One salient feature of the data in Figure 2A,B is that spermine exerts a larger effect on the ζ potential when the {PS} and the surface potential are large. For example, the concentration of spermine required to reduce the ζ potential to half of its initial value is about 0.01 M for the 20:1 PC:PS vesicles, 0.001 M for the 5:1 PC:PS vesicles, and 0.0001 M for the PS vesicles (Figure 2A,B). In other words, the apparent association constant, K_A (eq 1), increases as {PS} increases. This increase occurs primarily because the aqueous concentration of spermine at the surface of the membrane is enhanced by the Boltzmann term on the right-hand side of eq 1. For example, the surface potential of a 20:1 PC:PS membrane exposed to 0.01 M spermine is −4 mV, and the concentration of spermine in the aqueous phase at the surface of the membrane is enhanced by a factor of about 2. The surface potential of a pure PS membrane in 0.0001 M spermine is −30 mV (corresponding to a ζ potential of −23 mV; see Appendix), and theory predicts that the spermine concentration at the surface is enhanced by a factor of about 100. A less important reason for the increase in the association constant is that higher order complexes form more readily when the {PS} is high.

The curves predicted by the Gouy–Chapman–Stern theory describe a second important feature of the data: the slope of the ζ potential vs. log [S] curves. By comparing the dashed screening curve with the other curves in Figure 2C, which include spermine binding, we see that the predicted slope is essentially independent of the degree of spermine binding and is about 11 mV/decade of spermine concentration. The experimental results (Figure 2A,B) are in good agreement with this prediction.

A third feature of the data described by the theory is the shift of $[S]^{rev}$ to lower concentrations as {PS} increases. We assumed that both 1:1 and 1:2 complexes form between spermine and PS. Equation 6 predicts that the number of 1:2 complexes is proportional to $\{PS\}^2$ and is small when {PS} is low. As {PS} increases, $[S]^{rev}$ is predicted to decrease significantly, to 0.01 M with 1:1 PC:PS vesicles and to 0.005 M with PS vesicles (Figure 2C). These predictions correlate reasonably well with the experimental observations (Figure 2A),

although the interpretation is not unique.

(B) Gentamicin. We obtained similar ζ potential results with spermine and gentamicin. As illustrated in Figure 3A, gentamicin does not adsorb significantly to vesicles formed from PC at the concentrations we have examined: the ζ potential remains zero at all gentamicin concentrations. Gentamicin does change the ζ potential of vesicles that contain PS: when the {PS} is low (Figure 3B), the curves extrapolate to zero ζ potential when [gentamicin] = 0.03 M, and we calculate a 1:1 gentamicin–PS association constant of about 10 M^{-1}, the same values we obtained for spermine. The slopes of the curves in Figure 3A,B (12 mV/decade of gentamicin concentration) approximate the value of 11 mV/decade predicted by the Gouy–Chapman–Stern theory (Figure 2C,D). Finally, the concentration of cation required to produce charge reversal decreases as {PS} increases; this suggests that one gentamicin molecule can bind to more than one PS molecule. However, as illustrated in Figures 2A and 3A, the concentration of tetravalent cation required to reverse the charge of the PS vesicles shifts much more for gentamicin than for spermine as {PS} increases from about 1/140 $Å^2$ (1:1 PC:PS vesicles) to 1/70 $Å^2$ (PS vesicles). We cannot fit these gentamicin results by assuming that only 1:1 and 1:2 complexes form. We attempted to describe this shift theoretically by invoking higher order (1:3 and 1:4) complexes but still could not fit the data in both panel A and panel B of Figure 3 with a self-consistent set of binding constants. The adsorption of gentamicin to bilayer membranes is more dependent on the surface concentration of PS than predicted by our simple isotherms. A parsimonious interpretation of the data is that one gentamicin molecule binds to several PS molecules and that the strength of the cooperative binding is critically dependent on the surface density of the PS molecules in the membrane.

The number of gentamicin molecules adsorbed to a PS vesicle increases if {PS} is increased by replacing the unsaturated hydrocarbon tails of brain PS with saturated chains. We formed membranes from dimyristoylphosphatidylserine (DMPS), which is in the gel state at 25 °C (Hauser & Shipley, 1981). Gentamicin adsorbs about an order of magnitude more strongly to DMPS vesicles (triangles, Figure 3A) than to brain PS vesicles (circles). Resuls obtained by other investigators with unsaturated PC (area $\simeq$ 70 $Å^2$; Lewis & Engelman, 1983) and saturated PC (area $\simeq$ 50 $Å^2$; Lis et al., 1982; McDaniel et al., 1983) membranes suggest that the molecular areas of brain PS and DMPS at 25 °C are probably about 70 and 50 $Å^2$, respectively. These estimates agree with the available X-ray data (Loosley-Millman et al., 1982; Hauser & Shipley, 1981).

We also wanted to know if the adsorption of gentamicin to PS membranes would decrease if we sonicated the vesicles and lowered {PS} in the outer monolayer. The area occupied by a lipid in the outer monolayer of a sonicated egg phosphatidylcholine vesicle is about 74 $Å^2$ (Huang & Mason, 1978); the area should be similar for sonicated brain PS vesicles. We studied the adsorption of gentamicin to sonicated brain PS vesicles by measuring the electrophoretic mobility of multilamellar PS vesicles mixed with sonicated vesicles. The mobility measurement allowed us to monitor the free concentration of gentamicin in the solution. Specifically, we added a known concentration of sonicated PS vesicles to a solution containing a low concentration of multilamellar vesicles and 1 μM gentamicin. The sonicated PS vesicles adsorbed some of the gentamicin and changed the mobility of the multilamellar vesicles. We then titrated in a sufficient quantity of

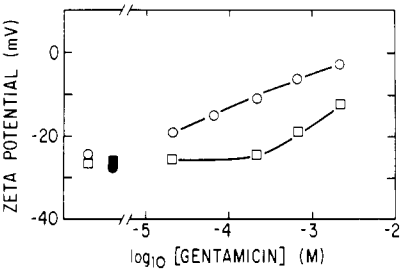

FIGURE 4: Effect of gentamicin concentration and pH on the ζ potentials of 5:1 PC:PS vesicles. The solutions contained 0.1 M NaCl and were buffered with 10 mM MOPS to either pH 5.0 (circles) or pH 7.4 (squares). The filled symbols indicate the effect of 100 μM EDTA. The curves have no theoretical significance.

gentamicin to return the free concentration of gentamicin in the solution to 1 μM, as determined from the mobility of the multilamellar vesicles. We knew the total concentration of gentamicin in the solution, and we deduced the number of gentamicin molecules bound to a PS molecule on the outer surface of a sonicated PS vesicle to be 0.06 ± 0.015 (n = 4). [The NMR measurements of McLaughlin et al. (1981) demonstrate that two-thirds of the lipids in a sonicated brain PS vesicle are in the outer monolayer]. We calculate from this measurement (see Discussion) that the association constant of gentamicin with sonicated PS vesicles is at least 1 order of magnitude lower than the association constant of gentamicin with multilamellar PS vesicles, a conclusion that agrees with the results of the NMR experiments on sonicated vesicles presented below.

We also investigated the effect of pH on the adsorption of gentamicin to membranes containing PS. When the pH of a 0.1 M NaCl solution containing 22 μM gentamicin was 7.4, 6.0, or 5.0, the ζ potential of a PS vesicle was −22.3 ± 0.8, −8.2 ± 0.6, or −6.5 ± 0.9 mV, respectively. The results obtained with 5:1 PC:PS vesicles at pH 5 and 7.4 are illustrated in Figure 4. Gentamicin reduces the ζ potentials of these vesicles about an order of magnitude more effectively at pH 5 than at pH 7.4. This result agrees with the observations of Laurent et al. (1982), who found that gentamicin binds more strongly to vesicles containing phosphatidylinositol (PI) at pH 5.4 than at pH 7.4.

We investigated the specificity of the interaction of gentamicin with monovalent negative lipids by studying the binding of the antibiotic to vesicles formed from PI and from mixtures of PI and PC. The results are very similar to those obtained with vesicles containing PS. Specifically, the charge of both PI and PS vesicles reverses at a gentamicin concentration of about 1 mM (Figure 3A,C). The slopes of the curves in Figure 3A,C are similar, about 12 and 10 mV/decade of gentamicin concentration, and agree well with the theoretical value of 11 mV/decade (Figure 2C). The value of $[\text{gentamicin}]^{rev}$ increases as the surface concentration of either PS (Figure 3A) or PI (Figure 3C) decreases, which indicates that gentamicin forms higher order complexes with both PS and PI. We conclude that the intrinsic 1:1 association constant is the same order of magnitude for PS and PI because the data in Figure 3D do not differ appreciably from the data in Figure 3B.

We studied the interaction of gentamicin with the mono-, di-, and trivalent gangliosides G_{M1}, G_{D1a}, and G_{T1}. We observed that gentamicin has a smaller effect on the ζ potentials of vesicles formed from mixtures of PC and these lipids (data not shown) than on the ζ potentials of vesicles of comparable electrophoretic mobility formed from mixtures of PC and an anionic phospholipid (Figure 3). These results suggest that

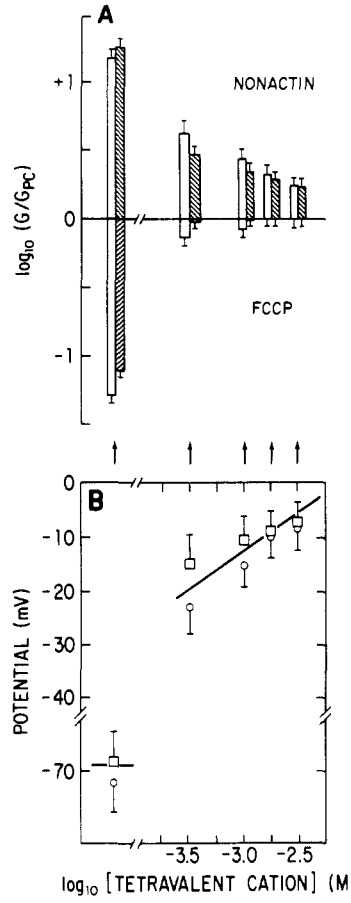

FIGURE 5: Effects of spermine and gentamicin concentrations on the surface potentials of 1:1 PC:PS planar bilayers. The solutions contained 0.1 M KCl and the indicated concentration of tetravalent cation and were buffered with 10 mM MOPS to pH 7.4. (A) Bar graph of bilayer conductance, G, measured relative to the conductance of a PC bilayer, G_{PC}, in the presence of 0.5 μM nonactin (upper bars) or 0.5 μM FCCP (lower bars). The open bars represent the effect of spermine concentration on the conductance; the hatched bars represent the effect of gentamicin concentration. (B) Surface potential as a function of spermine (squares) or gentamicin (circles) concentration, calculated from the data in (A) and eq 11. The line indicates the theoretical prediction of the Gouy–Chapman–Stern theory with the same intrinsic association constants used for spermine in Figure 2C.

gentamicin binds less strongly to gangliosides than to the negative phospholipids PS and PI.

We measured the effect of gentamicin on the ζ potential of brush border vesicles from rat renal proximal tubules. In a 0.15 M NaCl solution, pH 7.4, the ζ potential is initially -10.0 ± 1.0 ($n = 40$) mV. Addition of 3 mM gentamicin decreases the magnitude of the ζ potential by 2 mV to -7.6 ± 0.6 ($n = 30$) mV. Both the initial value of the ζ potential and the effect of gentamicin on the potential are comparable to the results obtained with 20:1 PC:PS vesicles (see Figure 3B).

Conductance Measurements. The conductance of a planar bilayer exposed to nonactin or FCCP is proportional to the equilibrium concentration of nonactin–K complexes or FCCP anions within the membrane. This concentration is an exponential function of the electrostatic potential within the membrane according to the Boltzmann relation. Thus, we can use conductance measurements to study the effects of spermine and gentamicin on the electrostatic potential within bilayers (McLaughlin, 1977). Figure 5A illustrates the effects of gentamicin or spermine on the conductance of 1:1 PC:PS planar bilayers formed in solutions containing 0.1 M KCl and either 0.5 μM FCCP (lower bars) or nonactin (upper bars). The bars at the left-hand side of Figure 5A illustrate that the FCCP conductance of a 1:1 PC:PS bilayer was 15-fold lower and the nonactin conductance was 17-fold higher than the FCCP or nonactin conductance of a PC bilayer. These symmetrical conductance changes imply that only the electrostatic potential changed upon incorporation of PS into a PC bilayer. Addition of 0.003 M spermine or gentamicin to the aqueous solution did not change the conductance of a PC membrane exposed to either probe, demonstrating that the polyvalent cations do not interact directly with the conductance probes. However, addition of 0.003 M spermine or gentamicin changed the conductance of a 1:1 PC:PS bilayer by about 1 order of magnitude (Figure 5A). Specifically, the nonactin conductance decreased 9-fold, and the FCCP conductance increased 15-fold. The slight asymmetry must be due to a change in some parameter other than the electrostatic potential, such as the membrane viscosity or dielectric constant. The measurements with the cationic and anionic probes were averaged, and the surface potentials calculated from eq 11 were plotted in Figure 5B. The initial value of the surface potential (−70 mV, Figure 5B) agrees with a measurement of the ζ potential of 1:1 PC:PS multilamellar vesicles (−55 mV; see Materials and Methods) if the plane of shear is 2 Å from the surface. The line in Figure 5B represents the prediction of the Gouy–Chapman–Stern theory, assuming that the intrinsic S–PS or G–PS 1:1 association constants are 10 M^{-1}, that the 1:2 association constants are 1000 M^{-1} Å^2, and that the potassium–PS association constant is 0.5 M^{-1} (see Materials and Methods). There is good agreement between the predictions of the theory and the results of both the conductance (Figure 5) and electrophoretic mobility experiments (Figures 2A and 3A). The electrophoretic mobility (ζ potential) depends only on the double layer potential in the aqueous phase, whereas the conductance responds to this potential and to any dipole or boundary potentials within the membrane (McLaughlin, 1977). Thus, our results argue against the suggestion of Brasseur et al. (1984) that gentamicin penetrates into the hydrocarbon region of the bilayer. This penetration would produce a large boundary potential within the membrane, which we would have detected with our conductance measurements.

NMR Results. (A) Sonicated Vesicles. We used ^{31}P NMR measurements to determine the change in the electrostatic potential at the phosphodiester group of PS upon addition of gentamicin. The data points in Figure 6A illustrate the effect of gentamicin on the line width of the ^{31}P NMR signal from the outer monolayer of sonicated PS vesicles when the solution contained 0.1 M NaCl and 0.2 μM free manganese. The points in Figure 6B illustrate the change in the electrostatic potential at the phosphodiester group, $\Delta\psi_p$, produced by gentamicin. They were calculated from the corresponding points in Figure 6A by using eq 12. The curve is the prediction of the Gouy–Chapman–Stern theory if we assume the association constant for the Na–PS complex is 1 M^{-1} (McLaughlin et al., 1981) and the 1:1 association constant for the gentamicin–PS complex is also 1 M^{-1}. The NMR results confirm the conclusion reached from microelectrophoresis measurements: gentamicin binds less strongly to sonicated PS vesicles than to multilamellar PS vesicles.

(B) Multilamellar Vesicles. The ^{31}P NMR spectrum of multilamellar PS membranes is shown in Figure 7A. The spectrum shows the characteristic asymmetric line shape with a pronounced low-field shoulder. The chemical shift anisotropy, which is sensitive to the details of the internal motion

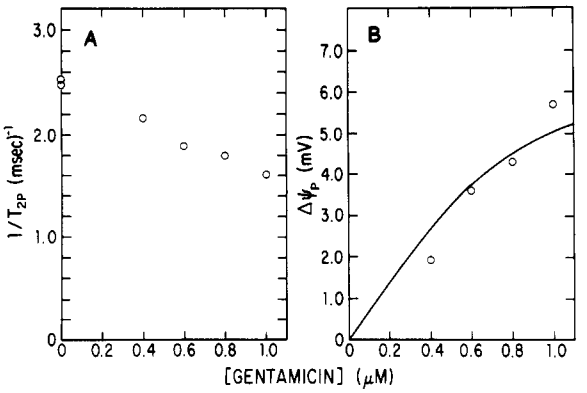

FIGURE 6: (A) Effect of gentamicin concentration on the ^{31}P NMR line width ($1/T_{2P}$) of PS molecules in the outer monolayer of sonicated PS vesicles. The solutions contained 0.1 M NaCl, 5 mM MOPS, and 0.2 μM free manganese, pH 7.4, T = 25 °C. (B) The change in the potential at the phosphodiester group, $\Delta\psi_P$, produced by gentamicin. $\Delta\psi_P$ was calculated from the data of (A) by using eq 12. The curve is the change in the surface potential predicted by the Gouy–Chapman–Stern theory, assuming that the intrinsic association constants for both the gentamicin–PS complex and the Na–PS complex are 1.0 M^{-1}.

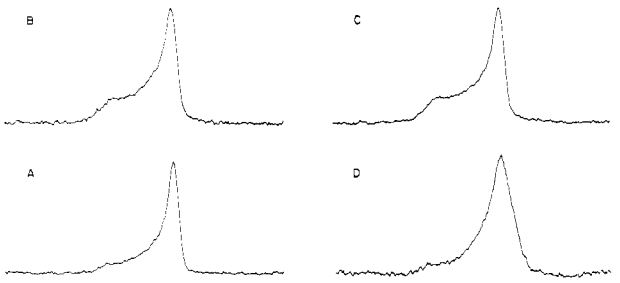

FIGURE 7: ^{31}P NMR spectra (145 MHz) of multilamellar PS vesicles. The aqueous solutions contained 0.1 M NaCl buffered to pH 7.4 with 5 mM MOPS. The total PS concentration was 15.5 mM. (A) No addition, T = 25 °C; (B) 1.4 mM gentamicin, T = 25 °C; (C) 7.5 mM Mg, T = 25 °C; (D) no addition, T = 5 °C. The total sweep width was 25 kHz.

of the phosphodiester group in the membrane (McLaughlin et al., 1975), was estimated from the separation between the inflection points on the high-field and low-field edges of the spectrum to be 57 ppm. Addition of up to 1.2 mM gentamicin (total concentration) to the 15.5 mM dispersion of PS did not affect the chemical shift anisotropy of the ^{31}P NMR signal. The only change was a slight increase in the height of the low-field shoulder, from 9% to 12% of the maximal spectral height (data not shown). However, increasing the gentamicin concentration from 1.2 to 1.4 mM increased the height of the low-field shoulder to 24% of the maximal spectral height (Figure 7B). This change was accompanied by a large increase in the turbidity of the solution, but no change in the chemical shift anisotropy was evident under these conditions. Increasing the gentamicin concentration from 1.4 to 5 mM caused no further changes in the spectrum. In particular, there was no sign of a "nonbilayer" form of lipid in the ^{31}P NMR spectrum (Cullis & McLaughlin, 1977), even at the highest gentamicin concentration.

The shape of the ^{31}P NMR spectrum in the presence of 1.4 mM gentamicin at 25 °C is not the same as the shape of the ^{31}P NMR spectrum observed for PS membranes at 5 °C (Figure 7D), which is slightly below the gel–liquid-crystalline phase transition for brain PS multilayers in 0.1 M NaCl (Jacobson & Papahadjopoulos, 1975). It is, however, exactly the same as the shape of the ^{31}P NMR spectrum of PS membranes in the presence of 7.5 mM $Mg(NO_3)_2$ (total concentration) at 25 °C (Figure 7C).

Discussion

We determined the association constants of spermine and gentamicin with negative lipids in a relatively model-independent manner. We circumvented the effects of electrostatic potentials by measuring the binding when the surface potential, charge density, and ζ potential are zero. For PS and PI, we deduced a 1:1 association constant of about 10 M^{-1} for both spermine and gentamicin.

We then tested the Gouy–Chapman–Stern theory by investigating the effects of these cations on the surface potential and ζ potential. The theory predicts that when the ζ potential is plotted against the log of the concentration of tetravalent cation, the maximum slope should be 11 mV/decade of concentration. The results obtained with spermine and gentamicin agree with this prediction. Equilibrium dialysis experiments designed to measure the binding of spermine to vesicles containing negative lipids are in progress in the laboratory of D. Papahadjopoulos (Meers et al., 1984). A comparison of these results with the electrostatic potential measurements reported here will provide a more rigorous test of the Gouy–Chapman–Stern theory for tetravalent cations.

We obtained most of our results from electrophoretic mobility measurements, which confirm and extend the measurements of Alexander et al. (1979). The calculation of the ζ potential from the electrophoretic mobility is, of course, subject to artifact. For example, the gentamicin and spermine molecules that adsorb to the membrane could decrease the mobility by extending from the surface and exerting a hydrodynamic drag. However, the conductance of a planar bilayer membrane does not depend on hydrodynamic effects: we used conductance measurements to estimate the effects of gentamicin and spermine on the surface potential of a planar bilayer membrane. The effects of gentamicin and spermine on the conductance are quantitatively consistent with the effects of these cations on the ζ potential. The conductance measurements strongly suggest that the calculation of the ζ potential from the mobility is a valid procedure, even when gentamicin and spermine bind to the membrane.

We were surprised that the effects of spermine and gentamicin on the potential could be described by the classical theory of the diffuse double layer, in which the ions are represented as point charges. These ions can be longer than the Debye length in a 0.1 M monovalent salt solution, which is 9.6 Å at 25 °C. We previously extended the classical theory to account for the finite size of large divalent cations in the aqueous diffuse double layer (Carnie & McLaughlin, 1983) and confirmed experimentally that when the size of a divalent cation is comparable to the Debye length the use of the classical theory is not appropriate (Alvarez et al., 1983). Why does the classical theory work for spermine and gentamicin? These cations change the electrostatic potential not by exerting a screening effect in the aqueous diffuse double layer [e.g., see McLaughlin (1977)] but by adsorbing to the membrane and changing the charge density. Furthermore, the slopes of the experimental curves in Figures 2 and 3 were determined most accurately for the PS and 1:1 PC:PS data, where the tetravalent cations interact with more than one negative lipid. We can explain our data most simply by postulating that the tetravalent cations lie flat on the surface when they bind to these membranes. Each charge would then sense the full electrostatic potential at the surface, the finite size would not be important, and the molecule would be electrostatically equivalent to a point tetravalent charge. We expect that the classical double layer theory will *not* account for the interaction

of large tetravalent cations with charged membranes if the cations do not lie flat on the surface when they adsorb. Indeed, when we studied the interaction of tetralysine with PS membranes in 0.1 M NaCl we observed that the slope of the ζ potential vs. $\log_{10}$[tetralysine] curve was not 11 but 23 mV/decade of concentration (data not shown). Similar deviations from the predictions of the theory were observed for tri- and pentalysine (data not shown). Our hypothesis about the orientation of adsorbed gentamicin and spermine molecules is clearly speculative.

Our experimental results suggest that gentamicin interacts more strongly with phosphate groups than with carboxylate groups on lipids. Specifically, the association constants of gentamicin with PS and PI are similar. Both PS and PI contain phosphate groups, but PS also contains a carboxylate group. Furthermore, gentamicin has a larger effect on the ζ potentials of vesicles containing a given mole fraction of the monovalent anionic lipid PI than G_{MI}. The charge on the phospholipid is on a phosphate moiety; the charge on the ganglioside is on a sialic acid (carboxylate) residue.

Our results demonstrate that association of gentamicin with membranes containing PS (and PI) increases markedly when the surface concentration of the negative lipid is greater than about one molecule per 100 Å^2. If we hypothesize (incorrectly) that gentamicin forms only 1:1 complexes with PS, then the Gouy–Chapman–Stern theory describes the data only if the intrinsic 1:1 association constant increases markedly as the surface concentration of PS increases. This hypothetical 1:1 association constant is of the order 1 M^{-1} for sonicated brain PS vesicles, 100 M^{-1} for multilamellar brain PS vesicles, and 1000 M^{-1} for multilamellar DMPS vesicles. Thus, the binding of gentamicin to vesicles formed from the negative lipid PS increases by about 3 orders of magnitude when the area occupied by PS decreases from about 75 Å^2 (outer surface of sonicated brain PS vesicle) to 50 Å^2 (multilamellar DMPS vesicles). Diluting the negative lipid PS with the zwitterionic lipid PC also reduces the adsorption of gentamicin to the vesicles. We were not able to explain the strong dependence of gentamicin binding on the surface concentration of PS (and PI) by extending the Langmuir isotherm to include higher order 1:2, 1:3, and 1:4 complexes. We consider it interesting that the interaction of a simple molecule with a phospholipid bilayer can be influenced markedly by slight changes in the molecular packing of the lipids.

The clinical utility of gentamicin is limited by its ototoxicity (Fee, 1980; Brummett, 1980) and nephrotoxicity (Smith et al., 1980; Plaut et al., 1979). Before it is taken up by the cells of the renal proximal tubule, gentamicin must interact with the brush border membrane. There is good evidence that gentamicin binds mainly to negative phospholipids in the brush border membranes (Sastrasinh et al., 1982). The negative lipids PS and PI comprise 20% and 7%, respectively, of the brush border phospholipids (Bode et al., 1976), but PS is probably located mainly on the cytoplasmic surface of the membranes (Chauhan et al., 1982). Just & Habermann (1977) and Sastrasinh et al. (1982) concluded that the apparent association constant of gentamicin with brush border membranes is about 40 000 M^{-1}. Although the intrinsic association constant of PI for gentamicin is only about 10 M^{-1}, this lipid could account for the binding observed to brush border membranes, as suggested by Sastrasinh et al. (1982), if the electrostatic potential adjacent to the phospholipid binding sites in their experiments was about −50 mV (see eq 1). The ionic strength was low, about 10 mM, in the experiments of Just & Habermann (1977) and Sastrasinh et al. (1982). The Gouy–Chapman–Stern theory predicts, and experiments confirm (McLaughlin et al., 1981), that the surface potential of a bilayer membrane containing 7% negative lipid is about −50 mV in a 10 mM salt solution.

Gentamicin might be transported into the proximal tubule cells by a receptor-mediated endocytotic mechanism that normally reabsorbs cationic polypeptides from the lumen (Just et al., 1977; Just & Habermann, 1977). This would account for the observation that gentamicin accumulates in the lysosomes (Just et al., 1977; Silverblatt & Kuehn, 1979; Morin et al., 1980). The nephrotoxicity exerted by gentamicin is associated with the appearance of excess lipid in the lysosome (Laurent et al., 1982). Kaloyanides & Pastoriza-Munoz (1980), Laurent et al. (1982), and Brasseur et al. (1984) discuss the possibility that gentamicin blocks the action of phospholipases in the lysosome. The pH in the lysosome is about 5, and lysosomal membranes isolated from rat kidney cortex contain 14% PI and 11% PS (Bode et al., 1976). Laurent et al. (1982) showed that the binding of gentamicin to membranes containing PI increases when the pH decreases. We studied the binding of gentamicin to bilayer membranes containing PS at pH 5 and 7.4 (Figure 4) and found, in agreement with their results, that gentamicin reduces the ζ potential about 1 order of magnitude more effectively at pH 5 than at pH 7.4. At pH 5, the ζ potential is zero when [gentamicin] = 3 mM (Figure 4), about the concentration inside a lysosome when the plasma concentration of gentamicin is sufficient to induce nephrotoxic effects in vivo (Laurent et al., 1982). There are at least two mechanisms by which gentamicin could inhibit the action of lipases. First, the gentamicin–phospholipid complexes could be less susceptible to degradation by phospholipases because of steric hindrance. Second, gentamicin can decrease the magnitude of the negative surface potential, which is required for the action of some lipases (Dawson, 1973; Irvine et al., 1979).

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APPENDIX

When the solution contains only monovalent cations and anions, the potential a distance x from the surface, $\psi(x)$, is

$$\psi(x) = \frac{2RT}{F} \ln \left[\frac{1 + \alpha \exp(-\kappa x)}{1 - \alpha \exp(-\kappa x)} \right] \quad \text{(A1)}$$

where

$$\alpha = \frac{\exp[F\psi(0)/(2RT)] - 1}{\exp[F\psi(0)/(2RT)] + 1} \quad \text{(A2)}$$

and $1/\kappa$ is the Debye length, where

$$\kappa = (2F^2 I \epsilon_r \epsilon_0 RT)^{1/2} \quad \text{(A3)}$$

$$I = (1/2)\sum_i c_i z_i^2 \quad \text{(A4)}$$

We know of no analytical description of the potential profile when the aqueous solution contains both 1:1 (e.g., NaCl) and 4:1 (e.g., spermine chloride) electrolytes. However, we can obtain an expression for the potential profile that is sufficiently accurate for our purposes by noting that when the surface and ζ potential are large [$\psi(0) > RT/(zF)$, [spermine] $< 10^{-3}$ M] the concentration of tetravalent cations in the aqueous phase is much lower than the concentration of monovalent salt ([NaCl] = 0.1 M). Thus, we may ignore the screening effects

of tetravalent cations in the diffuse double layer, which are illustrated by the dashed line in Figure 2C, and use eq A1 to describe the decay in potential with distance from the surface. When the concentration of tetravalent cations in the bulk aqueous phase increases to the point where they accumulate significantly in the diffuse double layer and change the Debye length (e.g., [spermine] > 1 mM), we note that the potential is not much larger than $RT/(zF)$ (see Figures 2 and 3). In this case, the Poisson–Boltzmann equation used in the Gouy–Chapman theory can be linearized without serious error, the theory reduces to the Debye–Hückel theory, and the potential predicted by eq A1 decays in an approximately exponential manner with distance, with a space constant equal to the Debye length defined in eq A3. Thus, we can use eq A1–A4 to describe the potential a distance x from the membrane over the entire range of spermine and gentamicin concentrations we investigated. The theoretical value of the ζ potential was obtained from the calculated surface potential by inserting x = 2 Å into eq A1.

Registry No. DMPS, 64023-32-1; spermine, 71-44-3; gentamicin, 1403-66-3.

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Evidence from ^{13}C NMR for Polarization of the Carbonyl of Oxaloacetate in the Active Site of Citrate Synthase†

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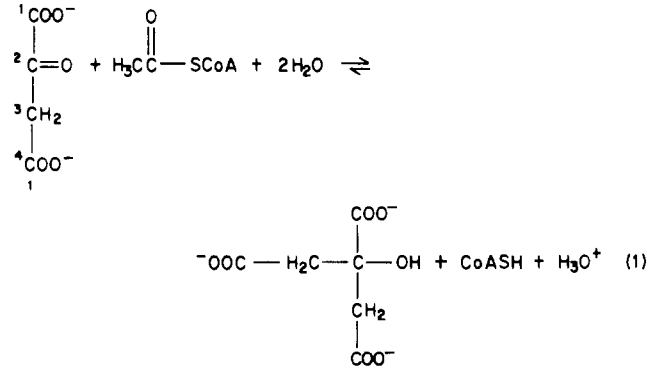
ABSTRACT: The carbon-13 NMR spectrum of oxaloacetate bound in the active site of citrate synthase has been obtained at 90.56 MHz. In the binary complex with enzyme, the positions of the resonances of oxaloacetate are shifted relative to those of the free ligand as follows: C-1 (carboxylate), −2.5 ppm; C-2 (carbonyl), +4.3 ppm; C-3 (methylene), −0.6 ppm; C-4 (carboxylate), +1.3 ppm. The change observed in the carbonyl chemical shift is successively increased in ternary complexes with the product [coenzyme A (CoA)], a substrate analogue (*S*-acetonyl-CoA), and an acetyl-CoA enolate analogue (carboxymethyl-CoA), reaching a value of +6.8 ppm from the free carbonyl resonance. Binary complexes are in intermediate to fast exchange on the NMR time scale with free oxaloacetate; ternary complexes are in slow exchange. Line widths of the methylene resonance in the ternary complexes suggest complete immobilization of oxaloacetate in the active site. Analysis of line widths in the binary complex suggests the existence of a dynamic equilibrium between two or more forms of bound oxaloacetate, primarily involving C-4. The changes in chemical shifts of the carbonyl carbon indicate strong polarization of the carbonyl bond or protonation of the carbonyl oxygen. Some of this carbonyl polarization occurs even in the binary complex. Development of positive charge on the carbonyl carbon enhances reactivity toward condensation with the carbanion/enolate of acetyl-CoA in the mechanism which has been postulated for this enzyme. The very large change in the chemical shift of the reacting carbonyl in the presence of an analogue of the enolate of acetyl-CoA supports this interpretation.

Citrate synthase (EC 4.1.3.7) catalyzes the condensation of oxaloacetate (OAA) with acetyl coenzyme A (acetyl-CoA) to form citrate (eq 1). The enzyme has been the subject of intense scrutiny for many years. Sufficient structural and kinetic data are available to suggest what catalytic strategies are used by this enzyme. The chemical mechanism is thought to involve generation of the carbanion (enolate) of acetyl-CoA which condenses with the carbonyl of OAA to form *S*-citryl-CoA as an intermediate (Eggerer, 1965; Weidman & Drysdale, 1979; Eggerer & Remberger, 1963; Bayer et al., 1981). It has been proposed that the carbonyl of OAA could



(1)

interact with an electrophilic residue, resulting in polarization of the C=O bond with substantial positive charge development at the carbonyl carbon (Srere, 1966). Polarization of the

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