Interaction of Ions and Water in Gramicidin A Channels

Streaming Potentials across Lipid Bilayer Membranes

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ABSTRACT For very narrow channels in which ions and water cannot overtake one another (single-file transport), electrokinetic measurements provide information about the number of water molecules within a channel. Gramicidin A is believed to form such narrow channels in lipid bilayer membranes. In 0.01 and 0.1 M solutions of CsCl, KCl, and NaCl, streaming potentials of 3.0 mV per osmolal osmotic pressure difference (created by urea, glycerol, or glucose) appear across gramicidin A-treated membranes. This implies that there are six to seven water molecules within a gramicidin channel. Electroosmotic experiments, in which the water flux associated with current flow across gramicidin-treated membranes is measured, corroborate this result. In 1 M salt solutions, streaming potentials are 2.35 mV per osmolal osmotic pressure difference instead of 3.0 mV. The smaller value may indicate multiple ion occupancy of the gramicidin channel at high salt concentrations. Apparent deviations from ideal cationic selectivity observed while attempting to measure single-salt dilution potentials across gramicidin-treated membranes result from streaming potential effects.

INTRODUCTION

Although electrokinetic measurements are a staple of the physical chemist's repertory, they have not, until now, been used to study the properties of ionic channels in bilayer membranes. Two major reasons for this lapse are: first, the effects are usually small and easily obscured by numerous artifacts; second, the interpretation of the data, assuming they are reliable, is generally ambiguous. For practical and theoretical considerations, however, we have undertaken electrokinetic measurements on membranes treated with the polypeptide antibiotic gramicidin A. The practical consideration was that, while trying to confirm the general belief that gramicidin A channels are ideally cationic selective at all salt concentrations, we found a decrease in single-salt diffusion potential values as a function of the absolute concentration of salt, and discovered that the apparent loss of selectivity at higher salt concentrations resulted from the presence of streaming potentials. Inasmuch as streaming

potentials were obviously large enough to interfere with these standard measurements, we felt a careful study of the phenomenon was warranted. The theoretical consideration was that the interpretation of streaming potential data becomes quite simple and rather interesting for a permselective channel which is so narrow that water molecules and ions cannot pass each other within it (single-file transport); namely, it is possible to determine the number of water molecules per channel (see Theory).

The gramicidin A channel is indeed permselective, and several lines of evidence suggest that transport through it approaches the ideal of a single-file mechanism. Its complete impermeability to $(\text{CH}_3)_4\text{N}^+$ (Myers and Haydon, 1972), and its permeability to H_2O but not to urea (Finkelstein, 1974 *a*) indicate the presence at at least one point in the channel of a cross section with radius $\leq 2\text{\AA}$. Model building (Urry, 1972) suggests that the gramicidin channel is a cylinder with radius $2\text{\AA}$ throughout its entire length. If this model is correct, and the channel walls are more or less rigid, then ions and water would necessarily undergo single-file transport.

This paper reports the results of streaming potential and electroosmotic measurements on gramicidin A-treated membranes, with an interpretation of the data which assumes the occurrence of single-file transport. The accompanying paper (Rosenberg and Finkelstein, 1978) considers water permeability data in relation to the electrokinetic results. Together, these studies present an intimate picture of water and ion movement through gramicidin A channels.

THEORY

There are several ways of seeing how, for a permselective pore in which water molecules and ions cannot pass one another, the number of water molecules in the channel is obtained from electrokinetic measurements.

Electroosmosis

The simplest approach is a direct consideration of electroosmosis. For a given number of moles of charge (coulombs) which pass through the membrane (current $\times$ time), a certain number of moles of water (volume) also crosses. Inasmuch as gramicidin A channels are ideally cationic selective (Myers and Haydon, 1972), all the current is carried by cations. If there is never more than one cation in a channel at any time, and if the ion cannot overtake water molecules in the channel, then for every ion crossing the membrane, all N water molecules within the channel must also cross. Thus, N is directly obtained from an electroosmotic experiment by dividing the number of water molecules that flowed across the membrane by the number of ions that produced this flow.

Streaming Potentials

Consider a gramicidin A-treated membrane separating identical solutions (called 1 and 2) of a uni-univalent salt (e.g., 0.1 M KCl). Let an impermeant nonelectrolyte, s , be introduced into compartment 2, and assume that the activity of the cation in 2 is not altered by this. (This point will be considered further in Results.) Normally, one would expect an osmotic flow of water from

compartment 1 to compartment 2 through the gramicidin channels.¹ But suppose that each channel always contains one cation. Then in an open-circuited situation there can be no osmosis, if single-file transport occurs, because charge would accumulate in compartment 2. In other words, the system is in thermodynamic equilibrium.²

For N water molecules per channel, the only kinetic unit which can cross the membrane is a cation in combination with N water molecules. Therefore, we can write for the equilibrium state:

$$N\mu_w(1) + \mu_+(1) = N\mu_w(2) + \mu_+(2), \quad (1)$$

where μ_w is the chemical potential of water and μ_+ is the electrochemical potential of the cation. Note that neither the chemical potential of water nor the electrochemical potential of the cation is equated, but rather a particular combination of the two. Under the conditions of our system, the "species" that is formally in equilibrium across the membrane is the cation hydrated by N water molecules. Eq. 1 can be expanded to:

$$g_1 NRT \ln X_w(1) + \mu_+'(1) + F\psi(1) = g_2 NRT \ln X_w(2) + \mu_+'(2) + F\psi(2), \quad (2)$$

where X_w is the mole fraction of water, μ_+' is the chemical potential of the cation,³ ψ is the electrostatic potential of a given solution, g is the rational osmotic coefficient (Robinson and Stokes, 1959), and R , T , and F have their usual meanings. Rewriting Eq. 2 we have:

$$[\psi(2) - \psi(1)] \equiv \Psi = N \frac{RT}{F} [g_1 \ln X_w(1) - g_2 \ln X_w(2)] + \frac{\mu_+'(1) - \mu_+'(2)}{F}. \quad (3)$$

The second term in Eq. 3 represents the contribution made to Ψ by the difference in cation activity on the two sides of the membrane. In principle this could be made zero by adjusting the concentration of the electrolyte on side 2 so that its activity equals that on side 1;⁴ in practice we correct for this term with a control experiment using valinomycin or nonactin (see Materials and Methods). The actual streaming potential, $\Psi_{\text{streaming}}$, comes from the first term. This can be written as

$$\Psi_{\text{streaming}} = N \frac{RT}{F} \left[\varphi_s \frac{n_s(2)}{n_w(2)} + 2\varphi_+(2) \frac{n_+(2)}{n_w(2)} - 2\varphi_+(1) \frac{n_+(1)}{n_w(1)} \right] = N \left(\frac{\bar{V}_w}{F} \Delta\Pi \right), \quad (4)$$

where φ_s and φ_+ are the molal osmotic coefficients of the nonelectrolyte and the electrolyte, respectively (Robinson and Stokes, 1959), n_i is the number of moles of i , $\bar{V}_w$ is the partial molar volume of water, and $\Delta\Pi$ is the difference in osmotic pressure. If the osmolality of the electrolyte is the same on both sides, i.e.,

¹ We neglect the flow through the bilayer proper, as this is irrelevant to these considerations.

² We thank Dr. D. G. Levitt for calling this to our attention and for suggesting the analysis presented in this section.

³ $\mu_+ = \mu_+' + F\psi$.

⁴ A good approximation of this occurs if the molality (rather than the molarity) of the salt is the same on both sides.

$$2\varphi_+(2) \frac{n_+(2)}{n_w(2)} = 2\varphi_+(1) \frac{n_+(1)}{n_w(1)},$$

or if

$$2 \frac{n_+(1)}{n_w(1)} \approx 2 \frac{n_+(2)}{n_w(2)} \ll \frac{n_s(2)}{n_w(2)},$$

then Eq. 4 simplifies to:

$$\Psi_{\text{streaming}} = N \frac{RT}{F} \varphi_s \frac{n_s(2)}{n_w(2)}. \quad (4 \ a)$$

Thus N , the number of water molecules in a channel, is directly determined from the magnitude of the streaming potential.⁵

Above, we assumed that all channels had one cation in them at all times. However, as long as a channel never contains *more* than one ion, the streaming potential is given by Eq. 4, regardless of the fraction of channels that are occupied by a cation at any instant. For even though osmosis of water occurs through the ion-free channels, the same arguments lead to Eq. (4) for the streaming potential of the ion-containing channels, and this potential is not significantly shunted by the ion-free channels.

Equivalence of Electroosmosis and Streaming Potentials

The ability to obtain the number of water molecules in a single-file channel from either electroosmosis or streaming potentials, entirely distinct entities, is a particular example of the general equivalence of the two phenomena. From irreversible thermodynamics one can write, for small gradients, the phenomenological equations (e.g., see de Groot, 1958):

$$I = L_{11}\Delta\Psi + L_{12}\Delta P, \quad (5 \ a)$$

and

$$J_v = L_{21}\Delta\Psi + L_{22}\Delta P, \quad (5 \ b)$$

where I is the current passing across the membrane (taken as positive when it passes from side 1 to side 2); J_v is the volume flow across it (in the same sense); ΔP is the pressure difference across the system⁶ (taken as positive when it is high on side 1); $\Delta\Psi$ is the electrical potential difference across the membrane (taken as positive when side 1 is positive); and the L 's are the so-called phenomenological coefficients. L_{21} and L_{12} are obtained from electroosmotic and streaming

⁵ For a 1-osmolal solution, $(\varphi_s n_s)/n_w = 1/55.6$; since at room temperature $RT/F = 25.6$ mV, this means that an osmotic gradient of one osmolal across the membrane produces a streaming potential of $N \times 0.46$ mV; i.e., each water molecule in the channel contributes approximately 0.5 mV, per osmolal gradient, to the streaming potential.

⁶ In experiments with lipid bilayers, it is not possible to apply significant hydrostatic pressure differences across the membrane. However, osmotic pressure differences, $\Delta\Pi$, which are readily applied, can be substituted in Eqs. 5 for ΔP with little error. (See Levitt et al. [1978] for a discussion of the exact form Eqs. 5 take when osmotic pressure differences, rather than hydrostatic pressure differences, act as a driving force.)

potential experiments, respectively. The Onsager reciprocal relations (see de Groot, 1958) guarantee, however, that

$$L_{12} = L_{21}. \quad (6)$$

Thus, if the number of water molecules in a very narrow channel is determined from an electroosmotic experiment, it is also determined from a streaming potential measurement by applying Eqs. 5 and 6, without reference to the derivation given above.

An alternative way of stating this is as follows:

Dividing Eq. 5 *b* by 5 *a* we have:

$$L_{21} = \left(\frac{J_v}{I} \right)_{\Delta P=0} L_{11}, \quad (7 \ a)$$

whereas from Eq. 5 *a* alone we have:

$$L_{12} = - \left(\frac{\Delta \Psi}{\Delta P} \right)_{I=0} L_{11}. \quad (7 \ b)$$

Applying the Onsager reciprocal relations, Eq. 6, to these equations we find that:

$$\left(\frac{J_v}{I} \right)_{\Delta P=0} = - \left(\frac{\Delta \Psi}{\Delta P} \right)_{I=0} \equiv \frac{\Psi_{\text{streaming}}}{\Delta P}. \quad (8)$$

For our experimental situation, we replace ΔP by $\Delta \Pi$, the osmotic pressure difference. Eq. 8 then becomes:

$$\Psi_{\text{streaming}} = \left(\frac{J_v}{I} \right)_{\Delta \Pi=0} \Delta \Pi. \quad (9)$$

Eq. 9 is a general result, independent of the nature of the pathway through which ions and water move. Furthermore, for dilute solutions, or for permselective pathways (independent of the concentration of the solution), J_v is the volume flow of water. Multiplying and dividing Eq. 9 by F and $\bar{V}_w$ we get:

$$\Psi_{\text{streaming}} = \left(\frac{F}{I} \frac{J_v}{\bar{V}_w} \right)_{\Pi=0} \left(\frac{\Delta \Pi \bar{V}_w}{F} \right) = N' \left(\frac{\bar{V}_w}{F} \Delta \Pi \right). \quad (10)$$

Eq. 10 is equivalent to Eq. 4; the second term in Eq. 10 is also the second term in Eq. 4, whereas the first term in Eq. 10 is N' , the number of water molecules transported per ion. Thus, in this general derivation, N of Eq. 4 is replaced by N' . N' can be determined either directly from an electroosmotic experiment or indirectly from a streaming potential experiment using Eq. 10. When, however, ion and water movement occur by a single-file process through a channel never containing more than one ion, N' also becomes N , the number of water molecules in a channel.

⁷ This is Saxén's law (see de Groot, 1958), established empirically before its theoretical derivation from Onsager's relations.

Although in theory N can be obtained either from electroosmotic or streaming potential experiments, in practice the former (*a*) are technically more difficult to perform, (*b*) are limited to high salt concentrations (to avoid prohibitively large voltages applied across the membrane), and (*c*) give spuriously high results because of unstirred layer problems, which generate an "ordinary" osmotic contribution to the volume flow. For these reasons we place more confidence in, and confine our presentation to, streaming potential experiments. Nonetheless, the electroosmotic results in 1 M KCl were only 30% larger than those derived from 1 M KCl streaming potential data.

MATERIALS AND METHODS

Membranes were made from a 2.5% solution of bacterial phosphatidylethanolamine (PE) in 2,2,4,6,6-pentamethylheptane. Bacterial PE was purchased from Supelco, Inc. (Bellefonte, Pa.); 2,2,4,6,6-pentamethylheptane from Analabs, Inc. (North Haven, Conn.). Valinomycin (val) was purchased from Eli Lilly & Co. (Indianapolis, Ind.); nonactin (non) was a gift from Miss Barbara Stearns of the Squibb Institute for Medical Research (Princeton, N.J.); phloretin was obtained from K and K Laboratories, Inc. (Plainview, N.Y.). Most of the gramicidin A experiments were with a sample obtained from the late Dr. Lyman Craig of The Rockefeller University. A few experiments were with gramicidin (gram) purchased from ICN Pharmaceuticals, Inc. (Irvine, Calif.), a mixture of 72% gramicidin A, 9% gramicidin B, and 19% gramicidin C (Glickson et al., 1972).

Streaming Potentials

Membranes were formed at about 23°C by the brush technique (Mueller et al., 1963) across a circular hole of 0.5 or 0.2 mm diameter in a Teflon or Saran Wrap partition separating two compartments each containing 3 ml of the appropriate solution; both compartments could be stirred with magnetic fleas. One compartment always contained a uni-univalent electrolyte (NaCl, KCl, or CsCl) at either 0.01, 0.1, or 1 M concentration. Three different techniques, all giving essentially the same results, were used to establish osmotic gradients. (*a*) The second compartment contained the same salt solution⁸ plus 2.0 M urea. By carefully adding the solutions to the two compartments and quickly "painting" the lipid mixture across the hole in the partition, we avoided significant intermixing of the two solutions. (*b*) The second compartment contained the same salt solution layered on top of either 8 M urea, 4 M glucose, or pure glycerol, with the level of the lower phase below that of the hole in the partition. After the membrane was formed, the osmoticant was mixed throughout the volume of the compartment; its final concentration was between 1.33 and 2.0 M.⁸ (*c*) The second compartment contained the same solution as the first. After the membrane was formed, the solution in the second compartment was replaced, using a perfusion pump, with one containing the same electrolyte at the same concentration⁸ plus either urea, glycerol, or glucose at a concentration of from 1.33 to 2.0 M.

When the membrane had formed (conductance $<10^{-10} \Omega^{-1}$), gramicidin A was added to one or both sides from stock methanol or ethanol solutions to a concentration of from 10^{-8} to 10^{-6} M. (The final concentration of methanol or ethanol never exceeded 0.3%; control experiments showed such concentrations to have insignificant effects on membrane conductance.) After the membrane conductance reached an appropriate value

⁸ The electrolyte concentrations were of either the same molality or the same molarity. The former is somewhat more appropriate, but, as we shall see, proper controls assured the validity of the measurements in either circumstance.

(10^{-7} to 10^{-2} mho), the membrane potential was recorded, the membrane then broken (by tapping the chamber), and the potential in the absence of the membrane recorded. Frequently, a new membrane was then formed, the contents of the compartments briefly stirred, and the potentials in the presence and absence of a membrane again recorded. This could be repeated three or four times without any significant differences in recorded potentials. (Apparently, there was insignificant intermixing of the solutions from one membrane to the next.) Similar procedures were followed with valinomycin or nonactin. For those nonactin experiments in which the electrolyte was NaCl, phloretin was also added to both compartments, from a stock ethanol solution, to a concentration of from 10^{-5} to 10^{-4} M, so as to achieve reasonably high conductances (Andersen et al., 1976). (The same amount of phloretin was added in the corresponding gramicidin experiment.)

The osmolality of the solution in each compartment was obtained from the Handbook of Chemistry and Physics, 57th edition. We assume that the osmolality of the salt solution containing the nonelectrolyte is given by the sum of the osmolalities of the corresponding separate salt and nonelectrolyte solutions.⁹

Membrane potential and conductance were continually monitored during an experiment. Two pairs of Ag/AgCl electrodes were used: one pair to pass constant current, the other to record the potential difference across the membrane (with and without current being passed). The current source was a battery (1.5–6 V) with a large (10^{10} – 10^6 Ω) resistor in series (Finkelstein, 1974 *b*). The recording electrodes were connected to a high ($>10^{12}$ Ω) input impedance amplifier, and its output was displayed on an oscilloscope screen. The membrane potentials (in the absence of a stimulating current) given in Table I and referred to throughout this paper are always the difference between the potential with the membrane present and that with the membrane broken. This procedure corrects for any contribution from the electrodes themselves to the measured potential, but possibly introduces small undetermined errors from liquid junction potentials with the membrane absent. No such errors occur, however, for the quantity of interest, the streaming potential (see Results). Except when otherwise indicated, all measurements were made in the absence of stirring.

Electroosmotic Measurements

The experimental arrangement was the same as that described previously for osmotic experiments (Holz and Finkelstein, 1970), except that there were two pairs of Ag/AgCl electrodes: one for passing current and one for recording potential. Membranes (1.3 mm² area) were formed at $\sim 23^\circ\text{C}$ in 1 M KCl; gramicidin was then added to the outer compartment. After membrane conductance reached a sufficiently high value (2×10^{-2} – 2×10^{-3} mho), a constant current (30–100 μA), measured with an ammeter in series with the membrane, was passed across the membrane (using the same circuitry as in the streaming potential experiments), and subsequent volume changes of the inner compartment were recorded as described previously for osmotic experiments (Holz and Finkelstein, 1970). The outer compartment was continuously stirred during the experiment.

RESULTS

The basic result, given in the last column of Table I, is that at 0.01 and 0.1 M CsCl, KCl, and NaCl the streaming potential, normalized to a 1-osmolal

⁹ This assumption might introduce a significant error in the 1.0 M salt experiments, since the total osmolality of the salt is comparable to that of the nonelectrolyte.

gradient, is 3.0 mV,¹⁰ with the compartment containing the nonelectrolyte positive with respect to the opposite side. At 1.0 M salt concentration, it is down to 2.35 ± 0.1 mV. In the remainder of this section, we consider the validity of these results, both as to their magnitude and to our interpretation of them as bona fide streaming potentials.

TABLE I
POTENTIALS PRODUCED BY OSMOTIC GRADIENTS ACROSS GRAMICIDIN-TREATED AND VALINOMYCIN- OR NONACTIN-TREATED MEMBRANES

Salt	Solute	Solute molarity	$\Delta\Pi$ (osmolality)	$\Psi(\text{gram})$	$\Psi(\text{val, non})$	Ψ^* streaming
		<i>M</i>	<i>osmol/kg</i>	<i>mV</i>	<i>mV</i>	<i>mV</i>
0.01 M‡CsCl	Urea	2.00	2.06	9.0	2.6	3.1
0.1 M‡CsCl	Urea	2.00	2.06	8.3	1.9	3.1
0.01 M‡KCl	Urea	2.00	2.06	7.4	1.0	3.1
0.01 M	Glycerol	1.37	1.58	5.5	1.0	2.8
0.1 M‡KCl	Urea	2.00	2.06	7.2	0.6	3.2
0.1 M‡	Urea	2.00	2.06	5.4§	-1.2§	3.2
0.1 M‡	Urea	2.00	2.06	5.8	-0.7	3.2
0.1 M‡	Urea	1.42	1.45	5.8	1.4	3.0
0.1 M	Glycerol	1.37	1.58	5.8	1.2	2.9
0.1 M	Glucose	1.63	2.14	9.6	3.6	2.8
0.01 MNaCl	Glycerol	1.37	1.58	5.8	1.3	2.8
0.1 MNaCl	Glycerol	1.37	1.58	6.4	1.6	3.0
1.0 M‡CsCl	Urea	2.00	2.32	5.5	0.1	2.3
1.0 M‡KCl	Urea	2.00	2.30	5.4	-0.4	2.5
1.0 M	Glycerol	1.37	1.62	4.7	0.8	2.4
1.0 MNaCl	Glycerol	1.37	1.61	5.8	2.2	2.2
1.0 M‡	Glucose	1.33	2.01	2.7	-1.7	2.2

* $\Psi(\text{gram}) - \Psi(\text{val, non})$ normalized to $\Delta\Pi = 1$ osmolal.

‡ Salt molarity was the same in both compartments. In all other experiments salt molality was the same.

§ Potential recorded with stirring on both sides of the membrane.

^{||} Membrane formed across 0.2 mm diameter hole. In all other experiments the hole diameter was 0.5 mm.

Consider the result obtained with gramicidin A-treated membranes separating the solutions: 0.1 M KCl//0.1 M KCl + 2 M urea. The measured membrane potential is +7.2 mV.¹¹ Before we assume that this measurement represents a streaming potential, we must consider two possible artifacts which may contribute to all or part of this value.

The first is that the activity of K^+ on the urea side is significantly lower than that on the opposite side, and hence the potential difference is simply a diffusion potential across the cation-permeable gramicidin A-treated membrane. The second is that, because of osmosis and the presence of unstirred

¹⁰ Streaming potentials were 3.15 ± 0.15 mV with urea as osmoticant, and 2.85 ± 0.1 mV with glycerol or glucose as osmoticant.

¹¹ Potentials are measured with respect to the side not containing the nonelectrolyte.

layers, the KCl concentration at the membrane surface is reduced on the urea side and elevated on the opposite side. Thus, the measured "streaming potential" is in reality a dilution potential arising from a difference in KCl concentration across the membrane proper.¹² (We can preclude the possibility that osmosis through the gramicidin channels produces such an artifact, for in that case the measured potential should increase with conductance [i.e., with the number of channels], whereas in fact it is constant between 10^{-7} and 10^{-4} mho. However, osmosis through the bilayer proper does create such an artifact [see below].)

The two artifacts just considered produce the same potential differences across valinomycin- or nonactin-treated membranes as across gramicidin-treated ones. On the other hand, inasmuch as the former antibiotics are carriers of unhydrated cations (Kilbourn et al., 1967; Pinkerton et al., 1969), no streaming potentials should be created by osmotic gradients across membranes treated with them (see Discussion). Thus, potentials recorded across valinomycin- or nonactin-treated membranes are excellent controls for those recorded across gramicidin-treated membranes¹³ and the actual streaming potentials are the differences between the two. For the case under consideration, the potential difference across gramicidin-treated membranes is +7.2 mV, and that across valinomycin-treated membranes is +0.6 mV.¹⁴ The actual streaming potential is therefore the difference between the two or 6.6 mV. (Because the osmolality of 2 M urea is 2.06, the streaming potential normalizes to 3.2 mV for a 1-osmolal gradient.)

Aside from the two artifacts just discussed, there may exist others we were not clever enough to consider. It is therefore reassuring that there is good agreement between two completely different sets of experiments: the electroosmotic and the streaming potential experiments. Namely, we find that Eq. 9 (or equivalently, the Onsager reciprocal relations) is satisfied. (See Theory.)

This rather lengthy analysis of the situation: 0.1 M KCl/0.1 M KCl + 2 M urea applies equally well to all other conditions of salt, concentration, and osmoticant¹⁵ under which we measured potential differences,¹⁶ and hopefully

¹² Wright and Diamond (1969) unfortunately refer to such dilution potentials measured across gall bladder as streaming potentials.

¹³ Valinomycin- or nonactin-treated membranes would also provide excellent controls for electroosmotic experiments on gramicidin-treated membranes. Unfortunately, the high conductances required for the experiments could not be obtained with these carriers, even on phloretin-treated PE membranes (Andersen et al., 1976).

¹⁴ This +0.6 mV is composed of contributions from the two artifacts described above. If the compartments are stirred, the potential declines to -1.2 mV, which is close to the -2 mV difference between potentials measured with a potassium-selective (Orion) electrode in 0.1 M KCl and in 0.1 M KCl + 2 M urea. (We thank Dr. Stuart McLaughlin for making this measurement for us.) Thus, the potential observed with stirring across a valinomycin- or nonactin-treated membrane results primarily from the difference in K^+ activity between the two solutions. The difference between the -1.2 mV potential observed with stirring and the +0.6 mV observed without stirring is due to the difference in dilution potentials for these two situations. These dilution potentials arise from the transmembrane salt concentration gradients that result from osmosis and the presence of unstirred layers. The unstirred layers are larger in the absence of stirring than during stirring, and hence the dilution potentials are larger in the former instance than in the latter.

¹⁵ In NaCl experiments, we did not use urea, because nonactin-treated membranes are so much

clarifies the entries in Table I. We reiterate that the entries under both " $\Psi(\text{gram})$ " and " $\Psi(\text{val, non})$ " are the differences in potentials with the membrane present and absent. Both, therefore, are in error by any liquid junction potentials that exist with the membrane absent. The entries in the final column, " $\Psi_{\text{streaming}}$ ", obtained by subtracting $\Psi(\text{val, non})$ from $\Psi(\text{gram})$, do not contain this error.

In the 0.1 M KCl experiments, the measured potential in gramicidin-treated membranes is constant between 10^{-7} and 10^{-4} mho. At higher conductances, however, the potential continuously increases with increasing conductance; concomitantly, stirring produces a larger decrease in this potential. For example, in the urea experiments just considered, the measured potential was +9.9 mV at a conductance of 3.7×10^{-3} mho, rather than +7.2 mV, and it decreased 3.5 mV rather than 1.8 mV with stirring. We attribute these effects at higher conductances to dilution potential contributions from osmosis through the gramicidin channels; they are consistent with the osmotic results in the following paper (Rosenberg and Finkelstein, 1978).

DISCUSSION

Validity of Streaming Potential Determinations

We have presented data on electrical potential differences across gramicidin A-treated membranes as well as valinomycin- or nonactin-treated membranes separating solutions of different osmolalities. Across the former occur genuine streaming potentials, whose magnitudes were determined from the differences between corresponding gramicidin-induced and valinomycin- or nonactin-induced potentials. Fundamental to this procedure is our assertion that no streaming potentials occur across valinomycin- or nonactin-treated membranes. There are compelling reasons for this assertion. (a) From what is known about valinomycin and nonactin complexes with alkali cations (Kilbourn et al., 1967; Pinkerton et al., 1969), it is most unlikely, a priori, that several water molecules are associated with them. (b) The same potentials are recorded across both valinomycin- and nonactin-treated membranes. If these are streaming potentials, then we must conclude that the same number of water molecules are associated with the transport of both ionophores, despite significant differences in their chemical structure and size. (c) Potentials recorded across membranes of 0.5 mm diameter are significantly different from those across membranes of 0.2 mm diameter (Table I). (We attribute this phenomenon to differences in boundary conditions.) Yet upon subtracting the valinomycin- or nonactin-induced potential from the gramicidin-induced potential, we obtain the same result for the two sizes of membrane. (d) Potentials across gramicidin-treated and valinomycin- or nonactin-treated membranes decrease significantly with

more permeable to NH_4^+ than to Na^+ , that the small NH_4^+ contamination of the urea solutions made these measurements meaningless as a correction for the potentials recorded across gramicidin-treated membranes.

¹⁶ Virtually all experiments were done at osmolality differences of between 1.45 and 2.3. We established, however, using 0.5, 1, and 2 M urea that streaming potential was a linear function of osmotic gradient.

stirring (Table I). Clearly, dilution potentials contribute to both measured potentials; the difference between them, however, remains constant. (e) Substantially smaller potentials occur across valinomycin-treated lecithin:cholesterol membranes (molar ratio 1:4 in membrane-forming solution) than across PE membranes. (E.g., in 0.1 M KCl with a 2.14 osmolal gradient of glucose, the valinomycin-induced potential is 1.0 mV across lecithin:cholesterol membranes and 3.6 mV across PE membranes.) The former membranes have a water permeability less than one-half that of the PE membranes (Finkelstein, 1976; Rosenberg and Finkelstein, 1978). All of these results are consistent with our belief that osmosis through the bilayer proper contributes to the measured potentials across both gramicidin-treated and valinomycin-treated membranes, and that by subtracting the latter from the former we obtain true values for the streaming potentials.

The Number of Water Molecules in the Gramicidin A Channel

A useful starting point for our present considerations is that ions and water molecules form a single file within the gramicidin A channel. Although this contention is not proved by the existing data, it is supported by the channel's known properties, particularly its permeability to water but not urea (Finkelstein, 1974a). Urry's (1972) plausible model in which the central pore is a cylinder of 2Å radius also demands single-file transport.

In 0.01 and 0.1 M CsCl, KCl, and NaCl, we obtain the same streaming potential of 3.0 mV per osmolal difference in nonelectrolyte concentration.¹⁷ This means (from Eq. 4 a) that 6.5 water molecules are transported per ion in a channel. If we assume that there is rarely more than one ion in a channel at these salt concentrations, we conclude that the gramicidin A channel contains 6–7 water molecules. (In the following paper we arrive at a similar conclusion in a completely independent manner.) This is a reasonable number. The $\Pi_{L,D}^6$ helix model for the channel (Urry, 1972), a cylinder 2Å in radius and 25–30Å in length, can accommodate about 10 water molecules.

In 1 M CsCl, KCl, and NaCl, the streaming potential per osmolal gradient is 2.35 mV (down from 3.0 mV in 0.01 and 0.1 M). The smaller streaming potential may indicate fewer water molecules in a channel (5.1 instead of 6.5), perhaps from a direct osmotic effect of the high salt concentration. Alternatively, at 1 M salt some fraction of the channels (at any instant) may contain two ions. Such channels would produce smaller streaming potentials,¹⁸ whose magnitudes depend on the location of the ions. The number of water molecules transported per ion is the average number of water molecules between ions, and this number determines the magnitude of the streaming potential in a doubly occupied channel.

¹⁷ Osmotic gradients in 0.01 M HCl produce no measureable streaming potentials. This is consistent with a Grotthus mechanism for proton transfer, as suggested (Hladky and Haydon, 1972) by the channel's "abnormally" large proton conductance. Little coupling of ion flow to water movement is expected with this conduction mechanism, because proton movement proceeds through the channel down a chain of water molecules, without pushing the water molecules along.

¹⁸ The parallel combination of singly and doubly occupied channels produces the macroscopically measured streaming potential.

Sandblom and his colleagues believe that the gramicidin channel has 4 ion-binding sites, with multiple ion occupancy occurring at relatively low ion concentrations (Sandblom et al., 1977; Eisenman et al., 1978; Neher et al., 1978). They concluded this from measurements of single channel conductances, I - V characteristics, and membrane potentials; no consideration was given to water molecules within the channel. Our present paper directs attention to this important facet of ion transport. To bring streaming potential data into harmony with other data, however, care must be taken to specify the depth of penetration of the ions into the channel and the placement of the water molecules among those ions.

The Effect of Deviations from Single File Transport

Deviations from rigid single-file movement of ions and water within the gramicidin channel affect the electrokinetic results. In electroosmosis, for example, an ion cannot traverse a single-file channel without pushing all the molecules ahead of it through the channel, and so N' (the number of water molecules transported per ion in an electroosmotic experiment) becomes N (the number of water molecules within the channel). If the channel is not a rigid single-file channel but occasionally allows an ion to slip around a water molecule, then N' underestimates the number of water molecules within the channel, and is therefore a lower limit for that number. Because conclusions from streaming potential and electroosmotic experiments are fundamentally identical, N' obtained from streaming potential measurements must also be a lower limit for the number of water molecules in a channel. Thus, our data indicate that a gramicidin channel contains *at least* 6–7 water molecules.

Possible Misinterpretation of Diffusion Potentials

Streaming potentials affect diffusion potential measurements in gramicidin-treated membranes. (This was one of the original considerations leading to the present study.) For a 2:1 concentration gradient of KCl across a gramicidin-treated membrane, the potential is a function of total KCl concentration (Table II). Thus, for 0.2 M KCl vs. 0.1 M KCl, the potential is 15.6 mV. As KCl concentration increases, the potential declines monotonically, reaching 10.6 mV

TABLE II
POTENTIALS PRODUCED BY 2:1 RATIOS OF KCl
CONCENTRATIONS* ACROSS GRAMICIDIN-TREATED
MEMBRANES

KCl concentrations	Membrane potential
<i>M</i>	<i>mV</i>
0.2:0.1	15.6
0.5:0.25	14.0
0.64:0.32	13.6
0.8:0.4	13.2
1.0:0.5	13.0
2.0:1.0	10.6

* The ratios of KCl activities vary from 1.88 to 1.98.

at 2 M KCl vs. 1 M KCl. Similar measurements on valinomycin-treated membranes, however, yield ~17 mV throughout the concentration range. The data in Table II appear to indicate that the gramicidin A channel is not ideally selective for cations, and that it has substantial anion permeability at higher salt concentrations. The error of this view is readily apparent, however, if the osmolality of the solutions is kept constant with an impermeant nonelectrolyte. Thus, the potential increases from 10.6 to about 17 mV for 2 M KCl vs. 1 M KCl, when 2 M urea is added to the 1 M KCl, thereby removing the osmotic gradient across the membrane. Smaller potentials at high KCl concentrations result not from anion permeability, but from streaming potential contributions to the overall potential difference.¹⁹

Note Added in Proof Levitt et al. (1978) conclude, from streaming potential data analogous to ours, that there are about 11 water molecules in a gramicidin A channel, whereas we conclude, both from our data and theirs, that there are about 6 or 7. Their value of 11 results from *not* subtracting the potentials recorded with osmotic gradients across valinomycin- or nonactin-treated membranes from the corresponding potentials recorded with osmotic gradients across gramicidin A-treated membranes. For the reasons given in the Discussion, we believe this subtraction is essential.

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REFERENCES

- ANDERSEN, O. S., A. FINKELSTEIN, I. KATZ, and A. CASS. 1976. Effect of phloretin on the permeability of thin lipid membranes. *J. Gen. Physiol.* **67**:749-771.
- DE GROOT, S. R. 1958. *Thermodynamics of Irreversible Processes*. North-Holland Publishing Company, Amsterdam. 185-189.
- EISENMAN, G., J. SANDBLOM, and E. NEHER. 1978. Interactions in cation permeation through the gramicidin channel. Tl, K, Cs, Rb, Li, H, and possible anion binding. *Biophys. J.* **22**:307-340.
- FINKELSTEIN, A. 1974 *a*. Aqueous pores created in thin lipid membranes by the antibiotics nystatin, amphotericin B and gramicidin A: implications for pores in plasma membranes. In *Drugs and Transport Processes*. B. A. Callingham, editor. Macmillan & Co., Ltd., London. 241-250.
- FINKELSTEIN, A. 1974 *b*. Bilayers: formation, measurements, and incorporation of components. *Methods Enzymol.* **32**:489-501.
- FINKELSTEIN, A. 1976. Water and nonelectrolyte permeability of lipid bilayer membranes. *J. Gen. Physiol.* **68**:127-135.
- FINKELSTEIN, A., and R. HOLZ. 1973. Aqueous pores created in thin lipid membranes by the polyene antibiotics nystatin and amphotericin B. In *Membranes. Lipid Bilayers and Antibiotics*. G. Eisenman, editor. Marcel Dekker, Inc., New York. **2**:377-408.

¹⁹ Potentials across nystatin-treated membranes also decline with increasing salt concentrations (Finkelstein and Holz, 1973). The nystatin channel, however, is clearly not ideally anion selective, and only a small part of the decline results from streaming potentials (Varanda and Finkelstein, unpublished observations).

- GLICKSON, J. D., D. F. MAYERS, J. M. SETTINE, and D. W. URRY. 1972. Spectroscopic studies on the conformation of gramicidin A'. Proton magnetic resonance assignments, coupling constants, and H-D exchange. *Biochemistry*. **11**:477-486.
- HLADKY, S. B., and D. A. HAYDON. 1972. Ion transfer across lipid membranes in the presence of gramicidin A. I. Studies of the unit conductance channel. *Biochim. Biophys. Acta*. **274**:294-312.
- HOLZ, R., and A. FINKELSTEIN. 1970. The water and nonelectrolyte permeability induced in thin lipid membranes by the polyene antibiotics nystatin and amphotericin B. *J. Gen. Physiol.* **56**:125-145.
- KILBOURN, B. T., J. D. DUNITZ, L. A. PRIDA, and W. SIMON. 1967. Structure of the K⁺ complex with nonactin, a macrotetrolide antibiotic possessing highly specific K⁺ transport properties. *J. Mol. Biol.* **30**:559-563.
- LEVITT, D. G., S. R. ELIAS, and J. M. HAUTMAN. 1978. Number of water molecules coupled to the transport of Na⁺, K⁺, and H⁺ via gramicidin, nonactin or valinomycin. *Biochim. Biophys. Acta*. In press.
- MUELLER, P., D. O. RUDIN, H. T. TIEN, and W. C. WESCOTT. 1963. Methods for the formation of single bimolecular lipid membranes in aqueous solution. *J. Phys. Chem.* **67**:534-535.
- MYERS, V. B., and D. A. HAYDON. 1972. Ion transfer across lipid membranes in the presence of gramicidin A. II. The ion selectivity. *Biochim. Biophys. Acta*. **274**:313-322.
- NEHER, E., J. SANDBLOM, and G. EISENMAN. 1978. Ionic selectivity, saturation and block in gramicidin A channels: II. Saturation behavior of single channel conductances and evidence for the existence of multiple binding sites in the channel. *J. Membr. Biol.* **40**:97-116.
- PINKERTON, M., L. K. STEINRAUF, and P. DAWKINS. 1969. The molecular structure and some transport properties of valinomycin. *Biochem. Biophys. Res. Commun.* **35**:512-518.
- ROBINSON, R. A., and R. H. STOKES. 1959. *Electrolyte Solutions*. Butterworth & Co. Ltd., London. 29-30.
- ROSENBERG, P. A., and A. FINKELSTEIN. 1978. Water permeability of gramicidin A-treated lipid bilayer membranes. *J. Gen. Physiol.* **72**:341-350.
- SANDBLOM, J., G. EISENMAN, and E. NEHER. 1977. Ionic selectivity, saturation and block in gramicidin A channels: I. Theory for the electrical properties of ion selective channels having two pairs of binding sites and multiple conducting states. *J. Membr. Biol.* **31**:383-417.
- URRY, D. W. 1972. Protein conformation in biomembranes: optical rotation and absorption of membrane suspensions. *Biochim. Biophys. Acta*. **265**:115-168.
- WRIGHT, E. M., and J. M. DIAMOND. 1969. An electrical method of measuring non-electrolyte permeability. *Proc. R. Soc. Lond. Biol. Sci.* **172**:203-225.