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NUMBER OF WATER MOLECULES COUPLED TO THE TRANSPORT OF SODIUM, POTASSIUM AND HYDROGEN IONS VIA GRAMICIDIN, NONACTIN OR VALINOMYCIN

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Summary

The number of water molecules (n) coupled to the transport of cations across lipid membranes was determined in two different wats: directly from the electro-osmotic volume flux per ion, and, by the use of Onsager's relation, from the open circuit streaming potential produced by an osmotic pressure difference. The results of the two approaches were in general agreement. Monoolein membranes were formed on the ends of polyethylene or Teflon tubing connected to a microliter syringe and the volume change necessary to keep the membrane at a fixed position was measured. It was necesary to make corrections for unstirred layer effects. The results for gramicidin were: $n \approx 12$ for 0.15 M KCl and NaCl, $n \approx 6$ for 3.0 M KCl and NaCl, and $n \approx 0$ for 0.01 M HCl. For nonactin, $n \approx 4$ for both 0.15 and 3.0 M KCl and NaCl. Valinomycin (for 0.15 M KCl) behaved like nonactin. It is shown that for a channel mechanism, in general, n is less than or equal to the number of water molecules in a channel that does not contain any cations. Thus, the n of 12 for the 0.15 M salts implies that the gramicidin channel can hold at least 12 water molecules. This places an important constraint on models of the channel structure. The n of 0 for HCl is consistent with a process in which protons jump along a continuous row of water molecules. The decrease of n with the 3.0 M salts may indicate that the channel becomes multiply occupied at high salt concentrations. The n of 4 for nonactin and valinomycin means that at least four water molecules are associated with the carrier · cation complex, probably in the interstices between the complex and the disordered lipid.

Introduction

The volume coupled to the transport of ions across artificial bilayer membranes can be directly determined from electro-osmotic experiments. This approach will be used in this paper to determine the number of water molecules (n) coupled to the movement of an ion either by a carrier (nonactin,

valinomycin) or a channel (gramicidin) system. The value of n has important implications about the mechanism of transport and structure of these simple systems. For example, it will be shown that from n one can determine a lower limit for the number of water molecules present in the gramicidin channel and, by implication, the size of the channel. Recently, Rosenberg and Finkelstein [1] have also measured n for gramicidin using a similar approach.

For the case where the molality of the electrolyte is the same on the two sides of the membrane, the coupling of the volume (J_v) and current (I) flux to the potential (ψ) and osmotic driving force ($\Delta\pi$) is described by the equations (see Appendix):

$$J_v = L_{11}\alpha\Delta\pi + L_{12}\Delta\psi \qquad \alpha = \frac{1+n}{n + \bar{V}_I/\bar{V}_w} \tag{1}$$

$$I = L_{21}\alpha\Delta\pi + L_{22}\Delta\psi \qquad n = J_w/J_c$$

where J_w and J_c are the molar water and cation flux, respectively, and $\bar{V}_I/\bar{V}_w$ is the ratio of the molar volume associated with the cation to that of the water. Since the systems studied here are almost perfectly cation selective, it can be assumed that the flux of the neutral salt is negligible. The equations differ from the classical electro-osmotic equations (see ref. 2) because of the presence of α which is a function of n. This modification is necessary due to the use of an osmotic driving force instead of the classical hydrostatic driving force. In most cases, $\bar{V}_I/\bar{V}_w$ is less than 1 (see Appendix) and n is large compared to 1 so that α is very close to one and the difference is insignificant.

Two different types of experiments were made. In the first, $\Delta\pi = 0$ and the electro-osmotic volume flux produced by passing a current was measured. From Eqns. 1 and 2 (see Appendix):

$$n + \bar{V}_I/\bar{V}_w = \frac{zF}{\bar{V}_w}\left(\frac{J_v}{I}\right)_{\Delta\pi=0} = \frac{zF}{\bar{V}_w}\frac{L_{12}}{L_{22}} \tag{2}$$

In the second type of experiment, the streaming potential that results from an applied osmotic pressure difference when $I = 0$ (open circuit) was measured:

$$\left(\frac{\Delta\psi}{\Delta\pi}\right)_{I=0} = -\frac{L_{21}}{L_{22}} \tag{3}$$

Using Onsager's relation ($L_{12} = L_{21}$), these two measurements can be related:

$$n + 1 = -\frac{zF}{\bar{V}_w}\left(\frac{\Delta\psi}{\Delta\pi}\right)_{I=0} \tag{4}$$

The purpose in determining n from both types of measurements is not to test the Onsager relation but rather to check the experimental measurements, particularly, the correction for unstirred layer effects.

Methods

A slightly modified version of the system of Holtz and Finkelstein [3] was used. The "front" chamber consisted of a cavity in a Teflon block that had a

clear lucite window as the front wall. The "back" chamber consisted of a polyethylene or Teflon tube (internal diameter 0.76 or 1.14 mm) that was pushed through a hole in the back wall of the Teflon block. The black lipid membrane was formed on the end of the tube. Input and output ports allowed the solution in the front chamber to be changed without disrupting the membrane. The tubing was connected to a syringe so that the volume of the back chamber could be controlled. The electrical potential was measured with a differential amplifier (Analog Devices, model 603 J) connected to Ag/AgCl electrodes that were in direct contact with the front and back chambers. All experiments were carried out at room temperature.

The membranes were formed either from glycerol monoolein (Nuchek Prep, Elysian, Minn.) or egg lecithin (Analabs) plus cholesterol (Sigma) in decane. Gramicidin (ICN Pharmaceuticals) was added directly to the membrane-forming solution. Equal concentrations of nonactin (a gift from Squibb and Ciba) or valinomycin (Sigma) were added to both bathing solutions. Usually enough antibiotic was added to produce a membrane resistance of about 10^3 Ω cm^2.

Electro-osmotic measurements. For these experiments it was important to keep the volume of the back chamber as small as possible so that volume changes due to temperature flucturations were not important. This was accomplished by using as the electrode in the back chamber a hollow Ag(AgCl) cylinder that was connected at one end to the tubing and at the other end to a 10 μl syringe. The total volume of the back chamber was about 15 μl. The microliter syringe was controlled by a micrometer (1 small division = $1.8 \cdot 10^{-3}$ μl). The volume flux was determined from the volume change necessary to keep the membrane in the tube at a fixed position as observed through a 35× microscope. Volume changes as small as $0.3 \cdot 10^{-3}$ μl could be reproducibly measured.

Streaming potential measurements. In order to produce an osmotic driving force, a solution made hyperosmotic by the addition of an impermeant (either glucose or urea) was perfused through the front chamber. The molality of the electrolyte was the same on the two sides of the membrane since the anhydrous impermeant was added directly to the electrolyte solution. A different procedure was used when HCl was the electrolyte. The addition of glucose to the HCl solution (pH 2) produced a slight fall in pH. This solution was diluted with water until the pH was equal to that of the solution without glucose. In this case, the activity of H^+ was the same on the two sides of the membrane unlike the case for Na^+ and K^+ in which the molality was the same on the two sides. As is shown in the Appendix, for the H^+ case (equal activity) the $(n + 1)$ term in Eqn. 4 is replaced by n. The osmotic pressure difference between the two solutions produced by the addition of glucose was 1.04 osmolar (referred to in the text as 1 osmolar) as determined with a freezing point osmometer. Urea was used as the impermeant in a few experiments and the results were not significantly different.

Permeability measurements. The volume flux (J_v) produced by adding an impermeant (c_i) to the front chamber was measured directly using the same apparatus that was used for the electro-osmotic experiments. This flux was related to a membrane permeability to water (P) defined by (A_m = membrane

area):

$$P = \frac{J_v}{\overline{V}_w A_m c_i} \tag{5}$$

Unstirred layer corrections

The unstirred layers in front of the membrane should be small because the membrane was formed as close as possible to the tube end which projected into the continuously perfused front chamber. However, the volume flux in the streaming potential experiments should raise the electrolyte concentration behind the membrane in the unstirred closed tube that constitutes the back chamber. This will have two effects. First, a potential (of the same sign as the streaming potential) will be produced by the cation concentration difference. Secondly, the electrolyte concentration difference will lower $\Delta\pi$ which will reduce the volume flow and the streaming potential (Eqn. 4). In the following analysis, the magnitude of the first effect will be determined in the limit where the concentration of the electrolyte is much less than the impermeant and the second effect becomes negligible. Then the general case in which the second effect may be important will be considered. It will be assumed that the unstirred layer in the front chamber is small relative to that in the back chamber and can be neglected.

If the electrolyte concentration is small compared to the impermeant, $\Delta\pi$, and therefore, the volume flux (J_v) will be constants. If it is assumed that there is no convective mixing and that radial diffusion is so fast that the electrolyte concentration (c) is a function only of the axial position (x), then the following differential equation applies:

$$\frac{\partial^2 c}{\partial x^2} - \frac{\overline{u}}{D}\frac{\partial c}{\partial x} - \frac{1}{D}\frac{\partial c}{\partial t} = 0 \; ; \quad \overline{u} = J_v/A \tag{6}$$

where $\overline{u}$ is the mean flow velocity (and is constant), A is the area of the tube and D is the electrolyte diffusion coefficient. For the case where $I = 0$, there is no electrolyte flux across the membrane and the boundary conditions are:

$$t = 0 : \quad c(x, 0) = c_0$$

$$x = 0 : \quad \overline{u}c - D\frac{\partial c}{\partial x} = 0 \tag{7}$$

The solution for the concentration at the membrane ($x = 0$) is (ref. 4, p. 388; this solution must be expanded to remove the indeterminancy that occurs with this boundary condition):

$$\frac{c}{c_0} = (1 + 2\tau)(1 + erf\, \tau^{1/2}) + \frac{2}{\sqrt{\pi}}\tau^{1/2} e^{-\tau} \; ; \quad \tau = \frac{\overline{u}^2 t}{4D} \tag{8}$$

Expanding Eqn. 8 for $\tau << 1$:

$$\frac{c - c_0}{c_0} = \frac{\Delta c}{c_0} = \frac{4}{\sqrt{\pi}}\tau^{1/2} + \text{terms of order } \tau \tag{9}$$

Assuming that the front chamber is well stirred ($c = c_0$), the membrane poten-

tial due to the unstirred layer (ψ_d) is given by ($\tau << 1$):

$$\psi_d = \frac{RT}{F} \ln \frac{c}{c_0} \approx \frac{RT}{F} \frac{\Delta c}{c_0} = S_0 t^{1/2} ; \quad S_0 = 2\bar{u}(\pi D)^{-1/2} RT/F \tag{10}$$

If the electrolyte concentration is not small, the build up of Δc will decrease $\Delta\pi$ which will reduce the streaming potential and the volume flux ($\bar{u}$). The total membrane potential (ψ) is the sum of the streaming potential (Eqn. 4) and the potential due to the concentration difference produced by the unstirred layer:

$$\psi = \frac{(n+1)}{F} \bar{V}_w \Delta\pi + \frac{RT}{F} \ln \frac{c}{c_0} \tag{11}$$

The osmotic pressure difference is the sum of the impermeant in the front chamber and the electrolyte concentration difference across the membrane:

$$\Delta\pi = RT(c_i + 2c_0 - 2c) = RTc_i \left(1 - \frac{2c_0}{c_i} \frac{\Delta c}{c_0}\right) \tag{12}$$

Substituting Eqn. 12 into Eqn. 11 and expanding ($\Delta c << c_0$):

$$\psi \simeq \frac{(n+1)}{F} \bar{V}_w (RTc_i) - \frac{RT}{F} [1 - 2(n+1) \bar{V}_w c_0] \frac{\Delta c}{c_0} \tag{13}$$

where the first term is the streaming potential (Eqn. 4) at $t = 0$. At early times Δc will be small compared to $\Delta\pi$ so that Δc in Eqn. 13 can be approximated by Eqn. 9 (see Eqn. 10):

$$\psi \simeq \frac{(n+1)}{F} \bar{V}_w (RTc_i) + S(c_0)\, t^{1/2} ; \quad S(c_0) = [1 - 2(n+1) \bar{V}_w c_0] S_0 \tag{14}$$

In a plot of ψ versus $t^{1/2}$, the streaming potential and n can be determined from the intercept at $t = 0$. Using this n and the slope (S), the value of S_0 (Eqn. 14) can be determined. The water permeability of the membrane (P) can then be determined from S_0 (Eqn. 10):

$$P = \frac{J_v}{\bar{V}_w A_m c_i} = \frac{A\bar{u}}{\bar{V}_w A_m c_i} = \frac{F A_T (\pi D)^{1/2} S_0}{2RT A_m \bar{V}_w \Delta c_i} \tag{15}$$

Finally, using Eqn. 14, an independent estimate of n can be determined from the change in slope with c_0.

For electrolytes other than KCl, the diffusion potential that results from the concentration gradients in the unstirred layer will also contribute to the total potential. These potentials should also grow as $t^{1/2}$ so that the extrapolation of the plot to $t = 0$ should still give the streaming potential and n. However, the slope will be altered by this effect so that Eqn. 10 is no longer valid. For this reason, the water permeability will be determined only for the experiments with KCl.

Unstirred layers also interfere with the electro-osmotic measurements. The membrane current tends to produce a build up of electrolyte on one side of the membrane and a depletion on the other. This electrolyte concentration difference produces an osmotic volume flux in addition to the volume flux that is directly coupled to the current (and should be used in Eqn. 2). The magnitude

of this effect depends on the transference number of the cation (t_+) in the bulk solution. If t_+ is close to one (mobility of the cation is much greater than that of the anion) then for each cation that crosses the membrane another cation will be transported to the membrane and a concentration difference will not develop. If t_+ is close to zero, then for each cation that crosses the membrane, an anion will be transported away from the membrane and the concentration difference will be doubled. For this reason, one would expect a larger unstirred layer effect for NaCl than for KCl. This unstirred layer effect decreases as the electrolyte concentration is raised. For example, if 10 water molecules are carried with each cation, then when the K^+ concentration was 1/10 that of water (5.5 M) each K^+ would carry enough water to prevent any change in concentration. We tried to minimize the effect of unstirred layers by passing the current for the shortest time in which we could make accurate volume measurements so that the concentration differences would not have time to build up. Also, we alternately passed the current in opposite directions so that the electrolyte build up and corresponding augmentation of flow that occurred in one period should reduce the flow in the next period. Averaging the flow in the two directions should cancel unstirred layer effects.

Results

Streaming potential measurements

Fig. 1 shows typical records of the change in potential after the solution in the front chamber is changed to a solution that has the same electrolyte molality plus 1 osmolar glucose: Fig. 1, top is for the case where the electrolyte is 0.15 M KCl and gramicidin is present; Fig. 1, center is for 0.15 M KCl and nonactin; and Fig. 1, bottom is for 0.01 M HCl and gramicidin. The potential changes with 0.15 M KCl consist of two different components: a rapid change with a time constant similar to that of the time it takes to change the solution in front of the membrane, and a slow change that results from the cation build up in the unstirred layer. The intrinsic potential difference between the solutions due to the presence of the impermeant in the front chamber (in the absence of a membrane) was determined from the potential after the membrane broke and was used as the zero reference (see Appendix). Fig. 2 shows a plot of the late (slow) component ($t > 3$ min) of this potential change versus $t^{1/2}$ for a representative set of experiments. The points can be fitted by a straight line as they should if this component results from an unstirred layer (Eqn. 14). The streaming potential was determined by extrapolating to the intercept at $t = 0$.

The number of water molecules (n) coupled to the movement of the cation was determined from the streaming potential (for K^+ and Na^+, Eqn. 4; for H^+, $n + 1$ in Eqn. 4 is replaced by n; see Appendix) and is listed in Table I for various experimental conditions using glycerol monoolein membranes. For the gramicidin channel. the value of n was 11.3 and 12.7 for 0.15 M KCl and NaCl, respectively. When the electrolyte concentration was raised to 3.0 M, the value of n fell to about 6 for both K^+ and Na^+. For H^+, n was not significantly different from zero. In the presence of nonactin, n was about 4 for both K^+ and Na^+ and was independent of the cation concentration. The results for 0.15 M KCl

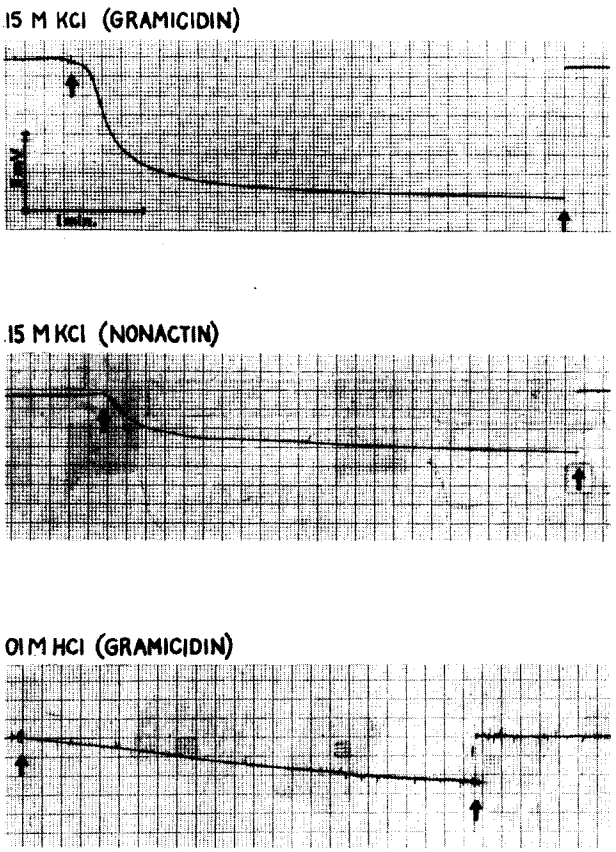

Fig. 1. Streaming potential experiments. Representative recordings of the change in membrane potential when the solution in the front chamber is switched to a solution with the same electrolyte molality plus 1 osmolar glucose. The first arrow indicates when the glucose solution first begins to pass the glycerol monoolein membrane. The second arrow indicates when the membrane breaks. Records are shown for 0.15 M KCl solutions with a gramicidin-doped membrane (top), 0.15 M KCl solutions with nonactin (middle) and 0.01 M HCl solutions with a gramicidin-doped membrane (bottom).

with valinomycin did not differ significantly from those with nonactin. The value of n was independent of the gramicidin or nonactin concentration over a range that corresponded to a 1000-fold change in resistance.

Membranes were formed on tubings of either 0.76 or 1.14 internal diameter. The value of n was independent of the tubing size but the slope was greater in the smaller tubing (Table I). This difference is probably the result of some convective mixing in the large tubing due to the presence of density gradients in the unstirred layers. For 0.15 M KCl with gramicidin, the permeability calculated from Eqn. 15 was 4.3 and $2.8 \cdot 10^{-3}$ cm/s for the smaller and larger tubing, respectively (Table I). In another set of experiments, the permeability was determined directly by measuring the volume flux produced by the osmotic pressure difference (Eqn. 5) and a value of $3.0 \pm 0.3 \cdot 10^{-3}$ cm/s was found; in rough agreement with the value determined from the slope. This agreement is evidence that the slow change in membrane potential in Fig. 1 is due to unstirred layers and that the extrapolation procedure used to determine

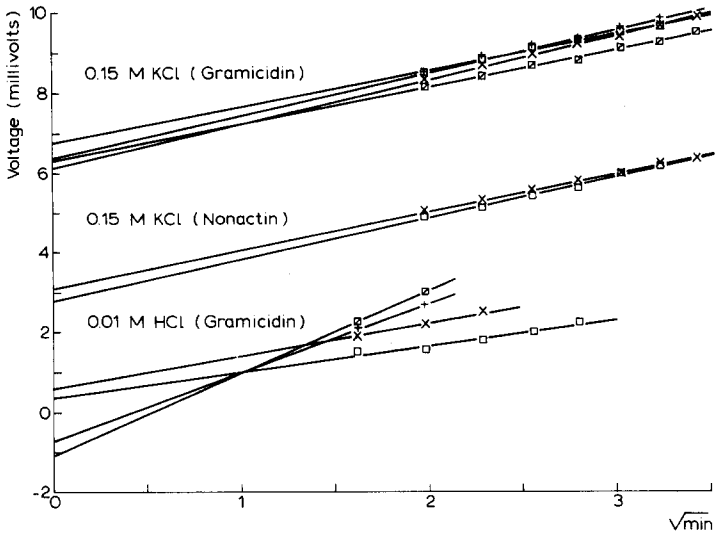

Fig. 2. Representative plots of the slow phase of the membrane potential change versus $t^{1/2}$ for the same experimental conditions as in Fig. 1. The lines are the least square fit to the data points. Each set of points represents a different experiment.

the streaming potential should be a good approximation. The water permeability was also measured directly with and without gramicidin and no difference was found; indicating that the intrinsic permeability of the monoolein membranes is so high that the water movement through the gramicidin channels cannot be detected. The slopes for NaCl and HCl cannot be related to the membrane permeability by Eqn. 15 because this equation neglects the diffusion potentials in the unstirred layers which chould be significant for these electrolytes but negligible for KCl.

Measurements were also made using lecithin (1%) plus cholesterol (1%) membranes with the hope that they would have significantly less water permeability and, therefore, smaller unstirred layer effects. The values of n for 0.15 M KCl with gramicidin was 11.6 ± 0.6 [4] which was not significantly different from that obtained with monoolein membranes (Table I). The slope (0.63 ± 0.08, internal diameter = 1.14 mm) was about 40% less than that for glycerol monoolein. The slope corresponds to a water permeability of $1.7 \cdot 10^{-3}$ cm/s which is in agreement with the value that was determined directly from volume flux measurements with these membranes ($2.4 \cdot 10^{-3}$ cm/s). This value is about four times higher than was found by Finkelstein [5] for membranes with a similar composition. Since this reduction in water permeability was not large, we continued to use monoolein membranes because we found them easier to work with.

The value of n can also be determined from the change in slope produced by a change in electrolyte concentration (Eqn. 14). The value of n at 3.0 M KCl (determined from the ratio of the slope with 3 M KCl to that at 0.15 M KCl) is about 7 for gramicidin and 5 for nonactin. These values are probably not very accurate because they depend on detailed assumptions about the unstirred layer effects. In contrast, the n determined from the streaming potential (inter-

TABLE I

STREAMING POTENTIAL MEASUREMENTS (mean ± S.E.)

	Electrolyte	Internal diameter of polyethylene tube (mm)	Intercept (mV)		n	Slope (mV/min$^{1/2}$)	P (10^{-3} cm/s)
Gramicidin	0.14 M KCl	0.76	5.7 ± 0.1	(6)	11.2 ± 0.2	1.60 ± 0.04	4.3 ± 0.1
	0.15 M KCl	1.14	5.8 ± 0.2	(15)	11.4 ± 0.4	1.03 ± 0.03	2.8 ± 0.1
	3.0 M KCl	0.76	3.2 ± 0.2	(3)	5.8 ± 0.4	0.09 ± 0.06	
	0.15 M NaCl	0.76	6.4 ± 0.2	(5)	12.7 ± 0.4	1.74 ± 0.07	
	3.0 M NaCl	0.76	3.3 ± 0.2	(2)	6.1 ± 0.4	0.30 ± 0.02	
	0.01 M HCl	0.76	−0.22 ± 0.41	(4)	−0.47 ± 0.8	1.3 ± 0.4	
Nonactin	0.15 M KCl	1.14	2.4 ± 0.2	(9)	4.1 ± 0.4	0.8 ± 0.1	2.3 ± 0.3
	3.0 M KCl	0.76	2.1 ± 0.2	(2)	3.5 ± 0.4	0.27 ± 0.03	
	3.0 M NaCl	0.76	2.6 ± 0.2	(3)	4.5 ± 0.4	0.43 ± 0.02	
Valinomycin	0.15 M KCl	1.14	2.5 ± 0.2	(4)	4.3 ± 0.4	1.01 ± 0.2	2.7 ± 0.5

cept) requires only a small correction for the unstirred layer and this correction involves only the assumption of the $t^{1/2}$ dependence. Thus, the agreement between the two sets of values at 3.0 M KCl is about as good as can be expected.

Electro-osmotic measurements

Fig. 3 (top) shows the change in volume when a current of 10 μA is passed for 3 min alternately in opposite directions in 0.15 M KCl solutions with a monoolein membrane containing gramicidin. Each volume measurement (solid circle) was determined by first turning the micrometer back a half turn and then turning it forward until the membrane came back to its initial position. Thus each measurement was independent and the scatter in the points during the control period when the current was off indicates that the error in the measurements was less than 10^{-3} μl. The value of n corresponding to the volume change during each three minute period was determined from Eqn. 2 (see Appendix for the value of $\overline{V}_I/\overline{V}_w$) and is shown in Fig. 3. The bottom of Fig. 3 shows a similar experiment with 3 M KCl and gramicidin and a current of ±20 or ±40 μA. The value of n was independent of current in the range from 10 (not shown) to 40 μA for 3 M KCl. The average values (± S.E.) of n determined from the electro-osmotic measurements were: 0.15 M KCl: 12.3 ± 0.8 (6); 3 M KCl: 6.6 ± 0.2 (10); and 3 M NaCl: 9.0 ± 0.7 (10). These values of n for KCl are not significantly different from those determined from the streaming potential (Table I). This agreement provides an additional check on these

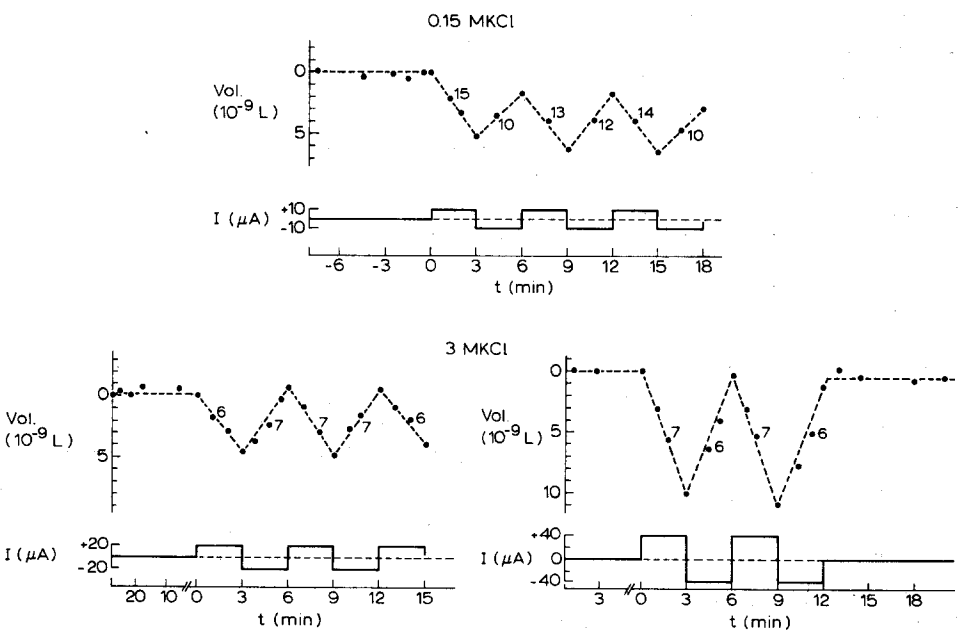

Fig. 3. Electro-osmosis experiments. Change in volume of the back chamber produced by an applied current for gramicidin-containing membranes. Beginning at $t = 0$, the current (bottom of each panel) was applied for 3-min periods, alternating in direction. The solutions contained either 0.15 M KCl (top) or 3 M KCl (bottom).

measurements and on the unstirred layer corrections. The unstirred layer effects were so large with 0.15 M NaCl that we could not make reliable measurements. Although these unstirred layer effects were greatly reduced with 3 M NaCl, they might still be the reason that the n for 3 M NaCl (9.0) is significantly larger than the n determined from the streaming potential (6.1).

Discussion

Gramicidin channel

The experimental measurement of the number of water molecules (n) coupled to the movement of a cation through the gramicidin channel is important because it can be shown that there is a general relationship between n and the number of water molecules (to be denoted by N) that are contained in the empty (no cation) channel. First consider the case in which the channel is so narrow (single file) that the cation and water cannot get around each other. Then, when the ion passes through the channel it will have to push out all the water molecules that were originally present in the channel (assuming that there is at most one ion per channel, see below) and $n \simeq N$. Even in this single file case there is some uncertainty about the relation between n and N because there is some evidence that the ion binds at the end of the channel by exchanging with the end water molecule [6]. In this case, n would be equal to N minus the two end water molecules or $N = n + 2$. Next, consider the case in which the channel is wide enough that the water molecules can get around the ion. Then, as the ion passes through the channel, some of the water molecules originally present will move around the ion and will not be displaced so that $N > n$. If there are two or more ions in the channel at a time, part of the volume displaced as the ion passes through the channel will consist of another ion, so that, again, $N > n$. Thus, in general, n is the minimum number of water molecules that must be able to fit in the channel.

The experimental value of $n = 11$—13 that was found for 0.15 M KCl and NaCl indicates that at least 12 water molecules can fit in the gramicidin channel. Urry et al. [7] have discussed a series of helical structures formed from dimers of gramicidin A which could form a stable channel. The structure which has been considered to represent the most probable model for the channel is the π^6 helix which has 6.4 residues per turn and is 25—30 Å long and 4 Å in diameter. Using a space filling (CPK) model of this channel, we found that the water molecules had to be in single file and that at most 10 water molecules could be placed in this channel. Considering the uncertainty in our experimental value of 12 water molecules per channel and the approximation involved in using the CPK models, our results are not incompatible with the π^6 helix. However, these results suggest that the π^6 helix is too small and that the next larger helix (π^8) which is 18—24 Å long and 6 Å in diameter may be the correct structure for gramicidin. A CPK model of this channel could hold 16—18 water molecules, a value in good agreement with our experimental finding of a minimum of 12 water molecules. Independent support for the larger helix comes from the observation [8] that organic cations that should be too large to pass through a 4 Å diameter channel (e.g. aminoguanidinium$^+$) have significant permeabilities through the gramicidin channel.

It has been shown [9,10] that H^+ has a very high conductance through the gramicidin channel. Myers and Haydon [10] suggested that there was a continuous row of water molecules in the pore and that the H^+ transport involved the same proton jump mechanism that occurs in the bulk liquid [11]. That is, a H^+ jumps on to a water molecule at one end of the channel and, after a series of rearrangements, another H^+ jumps off at the other end. One would predict for this mechanism that there would not be any coupling of water movement to this transport of H^+ and therefore n should equal zero, as was found experimentally.

The value of n for K^+ and Na^+ decreased from about 12 to 6 when the electrolyte concentration was raised from 0.15 to 3.0 M. One explanation is that the structure of the channel is altered by the high ionic strength. A more interesting possibility is that at high concentrations there is more than one ion per channel. If there are two ions per channel, n would be equal to the average number of water molecules between the two ions. If, as seems likely [6], the two ions are at the opposite extreme ends of the channel, n would only be decreased by (at most) one. Experimentally, the reduction at high concentrations was about 6. This result indicates either that when two ions are present they are not at the ends or that there are more than two ions present in the channel at concentrations of 3 M. Recently, several kinetic models of the gramicidin channel have been presented [6,12,13]. One of the features that varies in the different models is the number of ions that are in the channel at different electrolyte concentrations. A more detailed study of the effect of concentration on n should be able to distinguish between these models.

Levitt [14] has shown that if the channel is so narrow that the water molecules cannot get past each other, then it can be proved that, in general, the ratio of the osmotic to the diffusive permeability of water is equal to the number of water molecules in the channel (N). Since the present electro-osmotic measurements indicate that there are at least 12 water molecules per channel, the ratio of these two water permeabilities would have to be at least 12 if the channel were so narrow that two water molecules could not get past each other. Rosenberg and Finkelstein [15] have recently reported a value of about 5 for this ratio.

Nonactin and valinomycin

The structure determined by X-ray diffraction of the complex of K^+ with nonactin [16] or valinomycin [17] shows that the carrier forms a close fitting shell around the cation that should exclude water. In addition, NMR studies of the K^+ valinomycin complex in bulk phase C^2HCl_3 indicate that there are less than two water molecules per valinomycin molecule [18]. Therefore, one would not expect to find any water coupled to the transport of cations by these antibiotics and the finding of a significant streaming potential corresponding to an n of about 4 is a surprising result. It cannot be attributed to an intrinsic potential difference in the bulk solutions due to the addition of glucose since this amounts to less than 0.5 mV and remains after the membrane breaks (see Fig. 1 and discussion in Appendix). Nor can it be attributed to the build up of a polarization in the unstirred layers because it can be seen in Fig. 1 that, qualitatively, the time constant for the streaming potential (which is

related to the mixing time in the front chamber) is much shorter than the time constant for the unstirred layer effect which is described by Eqn. 13 and is similar for gramicidin, nonactin and valinomycin. Additional evidence that the streaming potential is not an artifact of the unstirred layer correction is the fact that no significant streaming potential is found for HCl with gramicidin although the unstirred layer effect is still present. We concluded from these arguments that there must be a direct coupling of water to the movement of the carrier · cation complex across the bilayer membrane.

The net flux of the carrier · cation complex is balanced by the flux of the unloaded carrier in the opposite direction, so that the value of $n = 4$ determined from the streaming potential means that, when the carrier moves across the membrane, the loaded carrier has four more water molecules coupled to it than does the unloaded carrier. Because the carrier fits very close to the unhydrated cation [14,15], the water must be external to the carrier. Space-filling models of the carrier complex in bilayer membranes [19] show that there are large distortions in the bilayer structure around the carrier in which water could be placed. This association of water with the carrier would increase the rate of transport across the membrane because it would raise the dielectric constant surrounding the carrier and, therefore, lower the electrostatic energy barrier for transport. The electrostatic calculation of Parsegian [20] shows that the energy barrier for the carrier · cation complex is very large (about 30 kT) so that just lowering the barrier by 20% (6 kT) will increase the transport rate by a factor of 400 (exp (6)). Even if the association with water was energetically unfavorable so that only a small fraction of the carrier · cation complexes had four water molecules associated with them, these associated complexes could still make up the majority of the transported ions because of their much faster rate of crossing the membrane. In contrast, the unloaded carrier would not see the electrostatic barrier and therefore the water-associated complexes would not have a faster transport rate.

Application to biological membranes

The analysis that has been developed here for gramicidin should be directly applicable to any cation or anion selective channel. Vargas [21] measured the streaming potential produced by internal perfusion of the squid axon with a solution made hyperosmotic with 0.3 M sucrose. Since K^+ was the only cation present, the value of n determined from the streaming potential (Eqn. 4) should be equal to the minimum number of water molecules in the K^+ channel. The water permeability of the squid axon [21,22] is less than one third of the permeability of the monoolein membranes that we used and therefore unstirred layer effects should be small. In addition, if the potential was due to unstirred layers, it should show a continual drift (with a time course of $t^{1/2}$) while the observed potential reached a definite plateau with a half time of about 2 min. (This half time is about what would be predicted for the diffusion of the sucrose to the cell membrane through the 200 μm shell of axoplasm that was left in the axon). If the 1.5 mV potential change produced by 0.3 M sucrose is a true streaming potential, then n for the K^+ channel (Eqn. 4) is about 10. This implies that the K^+ channel of the squid axon contains a minimum of 10 water molecules which is consistent with the channel diameter of 3 Å proposed by Hille [23].

Appendix

Derivation of electro-osmotic equations

The electro-chemical potential difference across the membrane for the cation is described by (assuming that n_c/n_w and $n_i/n_w << 1$):

$$\Delta\mu_c = RT \ln X_c^1/X_c^2 + \overline{V}_c \Delta P + zF\Delta\psi \tag{1A}$$

$$\frac{X_c^1}{X_c^2} = \frac{n_c^1}{n_c^1 + n_w^1 + n_i^1} \Big/ \frac{n_c^2}{n_c^2 + n_w^2 + n_i^2}$$

$$\approx \left(\frac{n_c}{n_w}\right)_1 \left(\frac{n_w}{n_c}\right)_2 \left[1 + \left(\frac{n_c}{n_w}\right)_2 - \left(\frac{n_c}{n_w}\right)_1 + \left(\frac{n_i}{n_w}\right)_2 - \left(\frac{n_i}{n_w}\right)_1\right] \tag{2A}$$

where X_c^1 and X_c^2 are the mol fractions of the cation on the two sides of the membrane and n_w, n_c and n_i are the number of mol of water, cation and impermeant (which includes the anion), respectively. For the experiments performed in this paper, the molality of the electrolyte is the same on the two sides so that $(n_c/n_w)_1 = (n_c/n_w)_2$ and:

$$\frac{X_c^1}{X_c^2} = 1 + \left(\frac{n_i}{n_w}\right)_2 - \left(\frac{n_i}{n_w}\right)_1 \approx 1 + \overline{V}_w \Delta m_i \tag{3A}$$

where $\overline{V}_w$ is the molar volume of water and Δm_i is the impermeant concentration (molality) difference across the membrane. Substituting Eqn. 3A into Eqn. 1A ($\overline{V}_w \Delta m_i << 1$):

$$\Delta\mu_c \simeq \overline{V}_w \Delta\pi + \overline{V}_c \Delta P + zF\Delta\psi \; ; \quad \Delta\pi = RT\Delta m_i \tag{4A}$$

Carrying out a similar procedure for water:

$$\Delta\mu_w = \overline{V}_w(\Delta\pi + \Delta P) \tag{5A}$$

Then, following the procedure of irreversible thermodynamics [1], the dissipation function is ($\Delta P = 0$):

$$\Phi = J_w \Delta\mu_w + J_c \Delta\mu_c \qquad \alpha = \overline{V}_w(J_w + J_c)/J_v$$

$$= J_v \alpha \Delta\pi + I\Delta\psi \qquad I = zFJ_c \tag{6A}$$

The linear equations are described by:

$$J_v = L_{11}\alpha\Delta\pi + L_{12}\Delta\psi$$

$$I = L_{21}\alpha\Delta\pi + L_{22}\Delta\psi \tag{7A}$$

Finally, the experimentally measured ratios are (assuming $L_{12} = L_{21}$):

$$\left(\frac{J_v}{I}\right)_{\Delta\pi=0} = \frac{\overline{V}_w J_w + \overline{V}_I J_c}{zFJ_c} = \frac{L_{12}}{L_{22}} \tag{8A}$$

$$= \frac{\overline{V}_w}{zF}(n + \overline{V}_I/\overline{V}_w) \; ; \quad n = J_w/J_c$$

and

$$\left(\frac{\Delta\psi}{\Delta\pi}\right)_{I=0} = -\frac{L_{21}\alpha}{L_{22}} = -\frac{L_{12}\alpha}{L_{22}}; \quad \alpha = \frac{1+n}{n + \overline{V}_I/\overline{V}_w}$$

$$= -\frac{\overline{V}_w}{zF}(1+n) \tag{9A}$$

where $\overline{V}_I$ is the volume associated with each mol of cations transported across the membrane. The value of $\overline{V}_I$ is determined from an analysis similar to that used in the measurement of transference numbers [24]. The volume that is measured is the total volume of the back chamber which includes the AgCl electrode. Thus, the total molar volume is:

$$\overline{V}_I = \overline{V}_{KCl} + \overline{V}_{Ag} - \overline{V}_{AgCl} \tag{10A}$$

where $\overline{V}_{KCl}$, $\overline{V}_{Ag}$, and $\overline{V}_{AgCl}$ are the molar volumes of the aqueous electrolyte, the silver and the silver chloride, respectively. Substituting the molar volumes [25] into Enq. 10A, one finds that $\overline{V}_I$ for KCl is 12 ml at 0.15 M and 14.7 ml at 3.0 M and $\overline{V}_I$ for NaCl is 2 ml at 0.15 M and 4.3 ml at 3.0 M. Since these values are less than $\overline{V}_w$ (18 ml) the ratio that appears in Eqn. 8A is less than 1.

Eqns. 8A and 9A are for the case where the molality of the electrolyte is the same on the two sides of the membrane. For the experiments with H^+ the pH on the two sides of the membrane was adjusted to the same value so that the activities are equal. This means that $X_c^1 \simeq X_c^2$ and the $\Delta\pi$ term does not appear in Eqn. 4A. Following the same procedure as above with this modified Eqn. 4A, it can be shown that the $(n + 1)$ term in Eqn. 9A is replaced by n and that Eqn. 8A is unchanged.

Effect of non-electrolyte on the membrane potential

Since the AgCl electrodes actually measure the Cl^- activity difference and not the membrane potential, one must consider the possibility that the streaming "potential" that is observed when glucose is infused into the front chamber might be due to an effect of glucose on the Cl^- activity. If glucose behaved ideally, it should produce a small potential change. It can be shown from Eqn. 1A that 1 M impermeant should produce a potential difference of $RT\overline{V}_w/zF \simeq$ 0.46 mV between two solutions that have the same Cl^- molality if there is no diffusion potential between the solutions (e.g. KCl). This was tested experimentally by measuring the potential difference between the two KCl solutions that were used in the streaming potential experiments (0.15 molal KCl in both solutions and 1 molar glucose in one solution). The potential was measured with Ag/AgCl electrodes and the solutions were connected with saturated KCl/agar bridges. A potential difference of about 0.5 mV was found, in good agreement with the theoretical prediction. In the streaming potential experiments, the potential just after the membrane broke was used as the zero reference. As is predicted theoretically, for KCl solutions this potential differed by about 0.5 mV from the initial potential when identical solutions were in both chambers. A small additional correction for the diffusion potential was made when NaCl (0.1 mV) or HCl (0.3 mV) was used as the electrolyte.

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References

1 Rosenberg, P.A. and Finkelstein, A. (1978) J. Gen. Physiol., submitted
2 Prigogine, I. (1967) Thermodynamics of irreversible processes, Interscience Publishers, New York
3 Holz, R. and Finkelstein, A. (1970) J. Gen. Physiol. 56, 125—145
4 Carlslaw, H.S. and Jaeger, J.C. (1959) Conduction of heat in solids, pp. 388—389, Oxford University Press, London
5 Finkelstein, A. (1976) J. Gen. Physiol. 68, 127—135
6 Levitt, D.G. (1978) Biophys. J. 22, 221—248
7 Urry, D.W., Goodall, M.C., Glickson, J.D. and Mayers, D.F. (1971) Proc. Natl. Acad. Sci. U.S. 68, 1907—1911
8 Eisenman, G., Krasne, S. and Ciani, S. (1976) in Ion and Enzyme Electrodes in Biology and Medicine (Kessler, M., Clark, L.C., Lübbers, D.W., Silver, I.H. and Simon, W., eds.), pp. 3—21, University Park Press, Baltimore
9 Hladky, S.B. and Haydon, D.A. (1972) Biochim. Biophys. Acta 274, 294—312
10 Myers, V.B. and Haydon, D.A. (1972) Biochim. Biophys. Acta 274, 313—322
11 Eisenberg, D. and Kauzmann, W. (1969) The structure and properties of water, Oxford University Press, New York
12 Sandblom, J., Eisenman, G. and Neher, E. (1977) J. Membrane Biol. 31, 383—417
13 Hladky, S.B., Urban, B.W. and Haydon, D.A. (1978) in Ion Permeation through Membrane Channels (Stevens, C.F., Tsien, R.W. and Chandler, W.K., eds.), in the press
14 Leviit, D.G. (1974) Biochim. Biophys. Acta 373, 115—131
15 Rosenberg, P.A. and Finkelstein, A. (1978) J. Gen. Physiol., submitted
16 Kilbourn, B.T., Dunitz, J.D., Pioda, L.A.R. and Simon, W. (1967) J. Mol. Biol. 30, 559—563
17 Duaz, W.L., Hauptman, H., Weeks, C.M. and Norton, D.A. (1972) Science 176, 911—914
18 Haynes, D.H., Kowalsky, A. and Pressman, B.C. (1969) J. Biol. Chem. 244, 502—505
19 Grell, E., Funck, T. and Eggers, F. (1975) in Membranes (Eisenman, G., ed.), Vol. 3, pp. 1—126, Marcel Dekker, Inc., New York
20 Parsegian, A. (1969) Nature 221, 844—846
21 Vargas, F.F. (1968) J. Gen. Physiol. 51, 123s—130s
22 Vargas, F.F. (1968) J. Gen. Physiol. 51, 13—27
23 Hille, B. (1975) in Membranes (Eisenman, G., ed.), Vol. 3, pp. 255—323, Marcel Dekker, Inc., New York
24 MacInnes, D.A. and Longsworth, L.G. (1932) Chem. Rev. 11, 171—230
25 Handbook of Chemistry and Physics (1971) The Chemical Rubber Co., Cleveland, Ohio