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Topical Review

The Gramicidin A Channel: A Review of Its Permeability Characteristics with Special Reference to the Single-File Aspect of Transport

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Summary. Gramicidin A forms univalent cation-selective channels of ~4 Å diameter in phospholipid bilayer membranes. The transport of ions and water throughout most of the channel length is by a single-file process; that is, cations and water molecules cannot pass each other within the channel. The implications of this single-file mode of transport for ion movement are considered. In particular, we show that there is no significant electrostatic barrier to ion movement between the energy wells at the two ends of the channel. The rate of ion translocation (e.g., Na^+ or Cs^+) through the channel between these wells is limited by the necessity for an ion to move six water molecules in single file along with it; this also limits the maximum possible value for channel conductance. At all attainable concentrations of NaCl, the gramicidin A channel never contains more than one sodium ion, whereas even at 0.1 M CsCl, some channels contain two cesium ions. There is no necessity to postulate more than two ion-binding sites in the channel or occupancy of the channel by more than two ions at any time.

I. Introduction

The problem of ion transport across cell membranes has concerned cellular and electrophysiologists for almost a century. Overton's recognition of the lipoidal nature of the plasma membrane (*see* Höber, 1945), which subsequently was attributed to the properties of the lipid bilayer that forms its basic structural feature (*see* Davson, 1962), posed the essential issue: how do small ions such as Na^+, K^+, and Cl^- traverse such a nonpolar structure? The idea that ions move through specialized pathways that function as either "carriers" or "pores" (also called "channels") eventually gained general acceptance among physiologists, and similar specialized pathways were also invoked for the permeation of polar nonelectrolytes across cell membranes (Höber, 1945).

Until about 15 years ago, the terms "carrier" and "pore" were generally used apologetically as only formal concepts that, at best, might be cartooned in a Discussion section. Today, the situation is different. The discoveries of molecules that function as carriers or pores in artificial lipid bilayer membranes (*see* Mueller & Rudin, 1969; Finkelstein & Mauro, 1977) and the isolation, characterization, and reconstitution of transport proteins from cell membranes (*see* Miller & Racker, 1979) have imparted molecular reality to these terms. It is now confidently felt that the transport of most ions and polar nonelectrolytes, including those whose transport is describable by carrier kinetics, occurs through proteinaceous pores (channels) spanning cell membranes (Singer, 1974; Rothstein, Cabantchik & Knauf, 1976).

The recognition of the reality of channels in biological membranes has intensified interest in the mechanisms of transport through them. An important manifestation of this interest has been the studies of the permeability properties of gramicidin A channels in lipid bilayer membranes. The gramicidin A channel has merited wide attention for several reasons: first, the gramicidin A molecule has a known, relatively simple structure. Second, the molecule is available in large quantities and can be chemically modified. Third, a reasonable molecular model of the channel exists, so that transport properties can be related to known structural features. Fourth, its ideal cationic selectivity and its intercationic specificities are relevant to many channels in biological membranes. Fifth, both the molecular model of the channel and the impermeability of the channel to small

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nonelectrolytes (e.g., urea) indicate that, in at least some region, transport should exhibit "single-filing" behavior, a subject of considerable biological and theoretical interest.

This article focuses primarily on the consequences and implications of single-file transport of water and ions through the gramicidin A channel. Our treatment of ion-transport and permeability is not exhaustive; indeed, to some our selection of topics and data may appear arbitrary and capricious. We feel, however, that the most useful contribution this article can make is to direct attention to some broad issues raised by ion-water interaction in a single-file channel. We hope this approach proves valuable both to the general reader interested in ion movement through very narrow channels and to investigators specifically engaged in the study of the gramicidin A channel.

II. Structure

Gramicidin A is a polypeptide antibiotic of known primary structure (Sarges & Witkop, 1965*a*, *b*). It has one of the most hydrophobic aminoacid sequences known (Segrest & Feldmann, 1974), consisting of fifteen hydrophobic aminoacids in an alternating L-D sequence (Fig. 1). The primary structure is remarkable, not only because of this aminoacid composition, but also because the N-terminal valine (the head) is blocked by a formyl group, and the C-terminal tryptophane (the tail) is blocked by an ethanolamine group. The molecule is thus entirely hydrophobic, apart from the carbonyl and imino groups of the peptide backbone and the formyl and ethanolamine end groups.

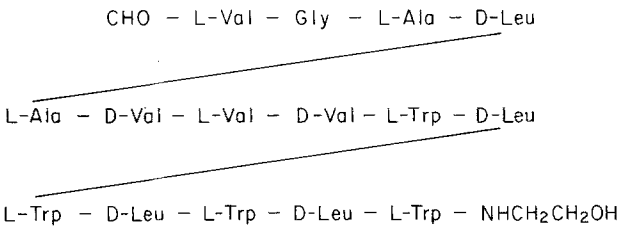

Fig. 1. Structure of valine-gramicidin A. Each horizontal row of amino acids corresponds to approximately one helical turn of Urry's β_6-helical model. The two diagonal lines represent peptide bonds connecting the three helical turns. The helix is stabilized by intramolecular hydrogen bonds connecting the amino acids obliquely; i.e., each amino acid in the channel interior is hydrogen-bonded to the two amino acids immediately adjacent to its vertical neighbors

Gramicidin A is very effective in artificial lipid bilayer membranes (Mueller & Rudin, 1967); submicromolar concentrations can increase membrane conductance more than 10^7 times above that of the unmodified bilayer. Membranes modified by gramicidin A are ideally permeable to small univalent cations (Myers & Haydon, 1972; Urban, Hladky & Haydon, 1980). It is well established that two gramicidin molecules dimerize to form a conducting pathway (Tosteson et al., 1968; Bamberg & Läuger, 1973; Zingsheim & Neher, 1974; Veatch et al., 1975; Veatch & Stryer, 1977), and that this pathway is a *channel* spanning the hydrocarbon part of the lipid bilayer (Hladky & Haydon, 1972). The compelling evidence for *channels* is the discreet conductance steps of uniform size (Fig. 2), seen in membranes treated with very small amounts of gramicidin A, the magnitude of which precludes a carrier-type mechanism (Hladky & Haydon, 1972).

There is substantial evidence that the channel is a hydrogen-bonded, head-to-head helical dimer (Bamberg, Apell & Alpes, 1977; Weinstein et al., 1979)[1], similar to the β-helical structures proposed by Urry (1971) and Ramachandran and Chandrasekaran (1972). According to this picture (Fig. 3), the polar groups of the peptide line the lumen of the channel, while the hydrophobic sidechains form the exterior surface of the channel, thereby making the structure compatible with the bilayer interior.

Direct structural characterization of the functional channel is rather limited. Two X-ray crystallographic structures, however, have been reported for gramicidin A dimers crystallized from organic sol-

[1] Gramicidin A exhibits considerable conformational heterogeneity in organic solvents. At least four different solution conformations can be identified (Veatch, Fossel & Blout, 1974; Veatch & Blout, 1974); some of these are dimers, but the relationship of any of these conformations to those of the channels in the lipid bilayer remains unclear.

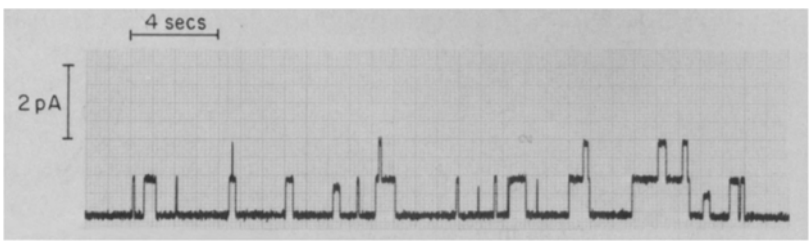

Fig. 2. Gramicidin A single channels. Note the rectangular current transitions and the predominance of one particular amplitude. The magnitude of the current steps, 1.0 pA, translates into a Na^+ flux through the channel of 6.3 $\times 10^6$ ions/sec—much too high for a mobile-carrier mechanism. Diphytanoylphosphatidylcholine/*n*-decane membrane; 0.1 M NaCl; 200 mV applied potential; $T = 25$ °C

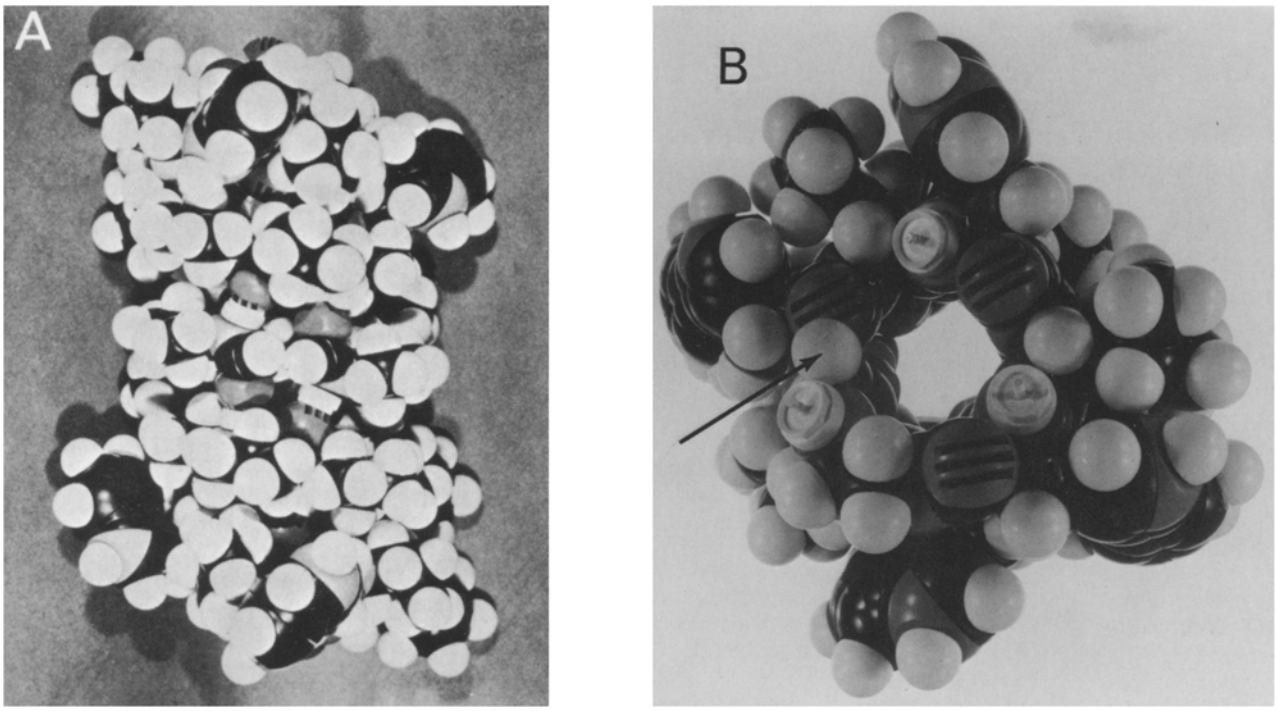

Fig. 3. Molecular model (CPK) of the gramicidin A channel in the left-handed β_6-helical form. In *A* the completely hydrophobic exterior surface is seen (from Urry, 1972); in *B* the channel has been turned 90° about a transverse axis to reveal the cylindrical lumen, lined by the polar groups (NH and C=O) in the peptide backbone. The transmembrane channel is a dimer of two gramicidin A molecules, joined by three hydrogen bonds at the formyl ends (*see* middle of *A*); the channel entrance is at the ethanolamine ends (seen in *B*). A sodium ion situated at its binding site in the gramicidin A channel, solvated between the carbonyl oxygen of tryptophan 11 and the hydroxyl of the terminal ethanolamine, is marked by an arrow. Note that it is possible for a water molecule to pass around a sodium ion sitting at this proposed location

vents (Koeppe, Hodgson & Stryer, 1978; Koeppe et al., 1979)—one for a cation-free gramicidin A dimer, and one for a structure composed of a gramicidin A dimer-cation (K^+ or Cs^+) complex. Both dimers are helical channels, but the binding of cations leads to a large conformational change: the diameter[2] of the cylinder formed by the atoms in the peptide backbone increases from about 5 to about 6.8 Å, and the length decreases from 32 to 26 Å. The complete structures have not yet been solved and may, for the reasons given in footnote 1, be of limited use for understanding ion permeation through the membrane-bound gramicidin A channel. Nonetheless, the properties of the gramicidin A-cation complex dimer (length: 26 Å, inner diameter $\approx$ 3.8 Å, and two symmetrical cation binding sites in the channel) are very similar to those predicted for the β_6-helical dimer (*see* Fig. 3)—the most likely conformation of the transmembrane channel (Urry et al., 1975). Further support for this conformation comes from an analysis of the current-voltage ($I-V$) characteristics of gramicidin A single channels (Andersen, Barrett & Weiss, 1981). This analysis positions the cation-binding sites in approximately the same places as the X-ray crystallographic analysis. Comparisons of single-channel data from gramicidin analogues indicate that the alkali metal cation-binding sites are located in the first helical turn of the channel, close to the aqueous phases (Andersen et al., 1981).

The gramicidin A channel is thus a very narrow structure, consisting of an array of coordination sites associated with the polar groups (in practice, the carbonyl oxygens) of the peptide backbone. An ion or a water molecule moves through this array by a series of rapid jumps from one site to another (or to the aqueous phases), with pauses of various durations at each site. The energy barriers and the number (and type) of particles at other sites in the channel determine both the probability of a particle being at a particular coordination site and the direction that particle moves in its next jump. For water, the energy

[2] These diameters refer to the average distance between the centers of the atoms, in the peptide backbone, on either side of the channel lumen. The space available for ion movement through the channel is about 3.0 Å smaller—two times the average van der Waals radius of the atoms lining the lumen. The effective luminal diameter for hydrogen-bonding solutes and water may be somewhat larger than this, as the distance between the donor and acceptor groups in a hydrogen-bonded pair may be less than estimated from their van der Waals radii [*see* Hille (1975*a*) for a further discussion of this subject].

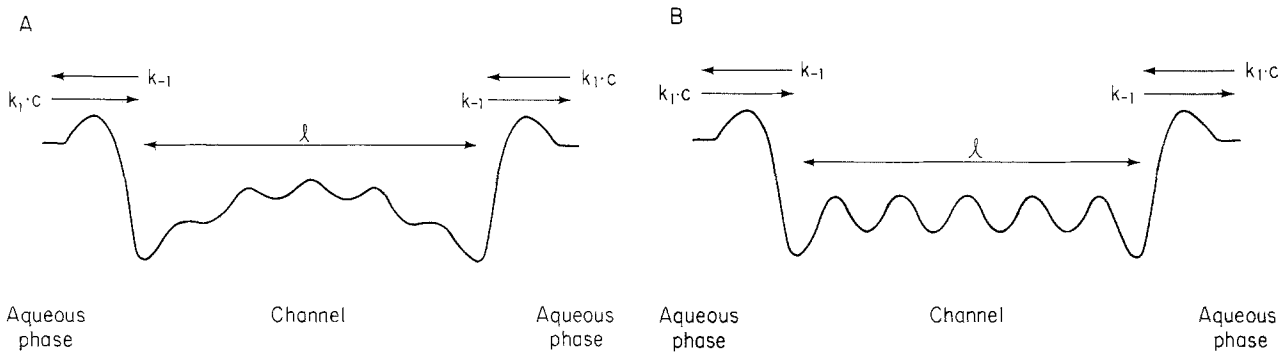

Fig. 4. Energy profiles for ion translocation through the gramicidin A channel. (The profiles have been drawn for a single ion moving through the channel and do not depict the changes which occur with multiple-ion occupancy.) (*A*): Energy profile with a central, electrostatic barrier. The short-range local interactions between the ion and the channel wall and the electrostatic repulsion of the ion from the channel interior combine to form two energy minima (two sites), one at each end of the channel, separated from each other (and from the aqueous phases) by energy barriers which the ion must traverse when moving through the channel [*see also* Levitt (1978) and Andersen and Procopio (1980)]. (*B*): Energy profile without a central, electrostatic barrier. The electrostatic barrier may be sufficiently small that ion movement through the channel becomes almost exclusively limited by the local interactions between the ion and the channel wall, as well as by single-file, flux-coupling to water. The energy profile will again show the same features as illustrated in *A*, but the physical meaning of the central barrier has changed considerably. The figures illustrate the various rate constants: k_1 is the rate constant for association of an ion with an empty channel ($k_1 \cdot c$ is the rate of ion entry); k_{-1} is the rate constant for dissociation of a singly-occupied channel; l is the rate constant for translocation through the channel interior. The association and dissociation rate constants for a second ion entering or leaving the channel are denoted in the text by k_2 and k_{-2}, respectively.

profile is presumably rather flat and therefore does not define any particular binding sites in the channel.

The energy profile traditionally drawn for an ion moving through the gramicidin A channel is illustrated in Fig. 4*A*. Note that there are no distinctive chemical groups (e.g., COO^-) in the channel which serve as major binding sites for the ion; the positions of the energy minima near the ends of the channel are therefore determined primarily by the superposition of local interactions between the ion and the channel wall and long-range electrostatic forces[3]. We argue later, however, that there is no significant energy barrier to ion movement within the channel, and that the energy profile is more realistically depicted in Fig. 4*B*.

III. Permeability Properties

The model of the gramicidin A channel described in the previous section has an internal diameter of approximately 4 Å. This value is consistent with the channel's permeability to H_2O but not to urea or larger nonelectrolytes (Finkelstein, 1974). The abnormally high proton conductance of the channel further suggests that the water molecules form a more or less continuous phase (Myers & Haydon, 1972), thus permitting a Grotthus, or "hopping", mechanism of transport characteristic of proton movement in bulk solution and accounting for its abnormally high mobility there. The 4-Å diameter of the channel both restricts this water phase to a single row and demands that ion and water movement occur by a single-file process; i.e., ions and water molecules cannot pass each other as they move through the channel. This single-file mechanism of transport has important consequences and implications, for both water and ion movement, upon which this article especially focuses. We first begin with water-water interaction and the general aspects of water-ion interaction in the channel. We then consider specifically the movement of Na^+ through the channel; this is a particularly simple case, since only one sodium ion occupies a channel at any instant, and consequently ion-ion interactions can be largely ignored. Finally, we examine the more complicated situation exemplified by Cs^+, in which ion-ion interactions must be treated because of multiple-ion occupancy.

A. Water Permeability

There are two coefficients that characterize the water permeability of any membrane. P_f, the osmotic (or filtration) permeability coefficient, is obtained from the flux of water resulting from an osmotic (or hydrostatic) pressure difference across the membrane:

$$P_f = \frac{\Phi_w RT}{A\Delta\Pi} \tag{1}$$

where Φ_w is the flux of water (in moles per unit time),

[3] The energy level of the minima are drawn below that of the ion in free solution, because univalent cations are favored in the channel relative to free solution (Myers & Haydon, 1972).

A is the membrane area, $\Delta\Pi$ is the osmotic pressure difference (created by a difference in concentration of impermeant solute on the two sides of the membrane), R is the gas constant, and T is temperature in degrees Kelvin. $P_d(H_2O)$, the diffusional permeability coefficient, is obtained from the flux of isotopically labeled water resulting from a concentration difference of labeled water (in the absence of any pressure difference):

$$P_d(H_2O)=\frac{\Phi^*}{A\Delta c^*} \tag{2}$$

where Φ^* is the flux of labeled water and Δc^* is the difference in concentration of labeled water on the two sides of the membrane. For a membrane consisting of channels through which water moves by a single-file mechanism,

$$\frac{P_f}{P_d(H_2O)}=N \tag{3}$$

where N is the average number of water molecules in a channel. This is a general result for single-file transport and is independent of any assumptions about the nature of the interactions of water molecules either with the walls of the pore or with each other (Levitt, 1974; Finkelstein & Rosenberg, 1979). For the gramicidin A channel, $P_f/P_d(H_2O)=5.3$ (Rosenberg & Finkelstein, 1978*b*), implying that there are 5-6 water molecules in the channel.

P_f and $P_d(H_2O)$ were determined in 0.01 M NaCl for phosphatidylethanolamine (PE) membranes containing many channels. Assuming no cooperative interactions among channels, one readily calculates the water permeability coefficients for a single channel, p_f and $p_d(H_2O)$, from the single-channel conductance in 0.01 M NaCl. These turn out to be (Rosenberg & Finkelstein, 1978*b*)

$$p_f=\frac{P_f A}{n}=9.58\times10^{-15}\ \text{cm}^3/\text{sec} \tag{4b}$$

$$p_d(H_2O)=\frac{P_d(H_2O)A}{n}=1.82\times10^{-15}\ \text{cm}^3/\text{sec} \tag{4b}$$

In 0.01 M NaCl

(n is the number of channels corresponding to a given value of P.) It is remarkable that the hydrodynamic permeability coefficient (p_f) of the very narrow gramicidin A channel, in which water molecules are probably present in a single-file array, agrees within a factor of 4 with the value predicted from Poiseuille's law, an equation derived from a macroscopic, continuum treatment of fluids (Finkelstein & Rosenberg, 1979).

B. Ion-Water Interaction

The coupling of ion and water fluxes in a channel is revealed by electrokinetic phenomena such as electroosmosis and streaming potentials. In the former, water is moved through the membrane when current flows in response to an applied potential difference; in the latter, a potential difference appears across the membrane when water flows in response to a hydrostatic or osmotic pressure difference. From either phenomenon one can always determine the number of water molecules transported per ion, but in the special case of an ion-selective channel through which single-file transport occurs, one can actually determine the number of water molecules (N) in the channel. This is most easily appreciated by considering an electroosmotic experiment at a salt concentration for which a channel contains no more than one ion. Then for every ion that flows across the membrane, all N water molecules in the channel must also cross. Thus, N is directly calculated by dividing the water flow by the current flow. Because of the equivalence of streaming potentials and electroosmosis as expressed through the formalism of irreversible thermodynamics (e.g., de Groot, 1958), N can equally well be determined from streaming potential experiments, which are technically easier to perform on planar lipid bilayer membranes than are electroosmotic experiments.

In 0.01 and 0.1 M salt solutions of NaCl, KCl, and CsCl, streaming potentials of 3.0 mV per osmolal of osmotic pressure difference are obtained across gramicidin-treated membranes; this means that 6.5 water molecules are transported per ion crossing the membrane (Rosenberg & Finkelstein, 1978*a*). At these concentrations there is at most one Na^+ in a channel at any time (*see* next section)[4]; therefore, 6.5 is the average number of water molecules per channel[5]. In 1 M salt solutions of NaCl, KCl, and CsCl, streaming potentials are reduced to 2.35 mV per osmolal gradient, which means that at these concentrations fewer water molecules (5.1 instead of 6.5) are transported per ion (Rosenberg & Finkelstein, 1978*a*). (*See* footnote 7 for a possible explanation.)

In summary, the number of water molecules in a gramicidin A channel has been determined both from

[4] At 0.1 M there is multiple-ion occupancy of channels by Cs^+ (and perhaps K^+). As these ions are situated at opposite ends of the channel (Andersen et al., 1981), it is not surprising that the electrokinetic results are the same as with Na^+.

[5] Levitt et al. (1978) conclude, also from streaming potential data, that the gramicidin A channel contains about 12 water molecules; Rosenberg and Finkelstein (1978*a*), however, discuss what they feel is an error in interpretation of the data that leads to this larger value.

water-water interaction [the ratio of P_f to $P_d(H_2O)$] and from ion-water interaction (streaming potentials). The two values, 5.3 and 6.5, are in good agreement, and we can reasonably conclude that there are about six water molecules in a channel. For a channel 4 Å in diameter and 25 to 30 Å in length (the working model for gramicidin A), each water molecule occupies a volume of 6×10^{-23} cc; that is, the density of water in the channel is about half that in bulk solution.

C. Results with Na^+

1. Single-Ion Occupancy

There are two principle reasons for believing that not more than one sodium ion occupies a gramicidin A channel at any instant. The first is that the shape of the single-channel conductance *vs.* sodium activity curve (Fig. 5) is associated with one-ion occupancy. That is, if we consider the reaction:

$$Na^+ + \text{channel} \rightleftarrows Na^+ - \text{channel} \tag{5}$$

and take the single-channel conductance (g_{Na}) proportional to [Na^+ − channel], the concentration of sodium-occupied channels[6], then the data in Fig. 5 are consistent with relation (6),

$$g_{Na} \propto \frac{[Na^+]}{K + [Na^+]} \tag{6}$$

$$K = \frac{[\text{channel}][Na^+]}{[Na^+ - \text{channel}]} \tag{7}$$

with $K = 0.31$ *m*; i.e., at an activity of 0.31 *m*, half the channels are occupied by one Na^+ and half are empty. This value of K is typical for gramicidin A channels in both phospholipid and glyceryl-monooleate (GMO) membranes.

Relation (6) predicts that g_{Na} should approach a limiting maximum value at large sodium concentrations. The data in Fig. 5 reflect this, but at very large sodium chloride concentrations (≈ 3.5 M), g_{Na} decreases slightly (about 10%) in phosphatidylcholine (PC) membranes (O.S. Andersen, *unpublished observations*). This might be taken as evidence for double occupancy of the channel by sodium ions at these concentrations. There is, however, another, more likely explanation for the decrease; namely, that it results from large osmotic pressures directly lowering g_{Na}. For example, g_{Na} in 1 M NaCl is lowered 23% by addition of 5 M urea to both sides of the membrane (O.S. Andersen, *manuscript in preparation*). A similar effect would be expected from large osmolarities of NaCl itself[7].

The second, and even more compelling, reason for believing that no more than one sodium ion ever occupies the gramicidin A channel is that the Behn-

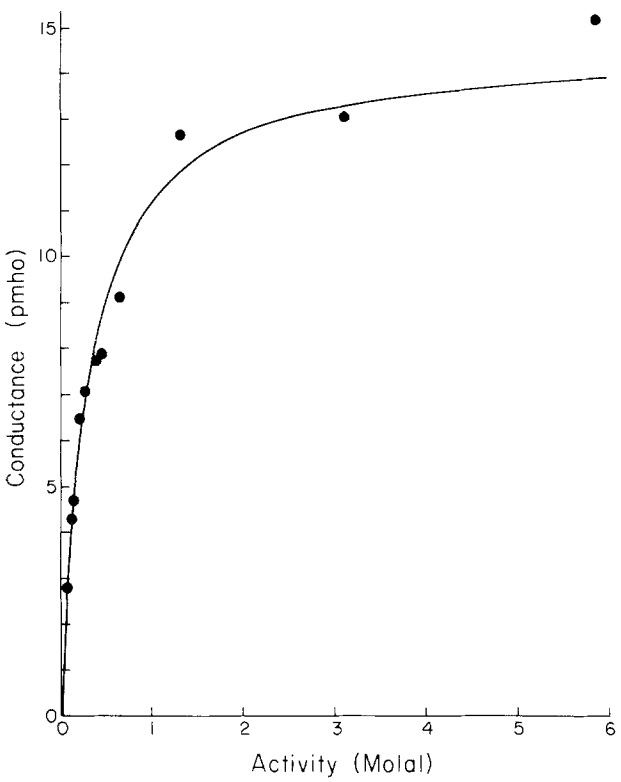

Fig. 5. Gramicidin A single-channel conductance as a function of the aqueous Na^+ activity. The curve is drawn according to relation (6), with $K = 0.31$ *m* and a maximum single-channel conductance of 14.6 pmho. [In terms of Eq.(19): $l = 2.0 \times 10^7$ sec^{-1}; $k_{-1} = 1.2 \times 10^7$ sec^{-1}; $k_1 = 1.9 \times 10^7$ m^{-1}.] The experimental points are from bacterial phosphatidylethanolamine/*n*-decane membranes, 100 mV applied potential, $T = 23$ °C

[6] The time-average conductance of a channel, which is what is always measured in gramicidin A single-channel conductance experiments, is proportional to the rate of ion movement through and out of the channel and to the fraction of time the channel has an ion in it. Assuming an Ergodic system, this latter quantity is proportional to [Na^+-channel].

[7] A possible origin for the effects of osmotic pressure on channel conductance is the large negative pressures generated within the pore when significant concentrations of impermeant solute are present on both sides of the membrane. In symmetrical solutions, the chemical potential of water within the pore must equal that in the external solutions. The addition of 5 M urea (or 2.5 M NaCl) to both sides of the membrane lowers the pressure within the pore (whatever that means in a single-file pore) by about 100 atmospheres from that existing in distilled water. The resulting stresses on the water molecules and the walls of the pore could alter channel conductance. For instance, it could reduce the number of water molecules in the pore, thus also accounting for the smaller streaming potentials at high salt concentrations.

Ussing-Teorell flux-ratio equation (Ussing, 1949; Teorell, 1953) is satisfied at all sodium concentrations from 0.1 to 5 M (Procopio & Andersen, 1979). That is, $n'=1$ in the equations:

$$\frac{\vec{M}_{Na}}{\overleftarrow{M}_{Na}}=e^{-n'F\Delta V/RT} \tag{8a}$$

and

$$\vec{M}_{Na}=\overleftarrow{M}_{Na}\equiv M_{Na}=\frac{RT}{n'F^2}G_{Na} \quad (\text{at } V=0) \tag{8b}$$

where, $\vec{M}_{Na}$ and $\overleftarrow{M}_{Na}$ are the unidirectional (tracer) fluxes of Na^+, ΔV is the applied transmembrane potential, F is the Faraday, and G_{Na} is the small-signal conductance of the membrane. Equation (8) has been discussed extensively for single-file processes, and it was in fact the observation by Hodgkin and Keynes (1955) that n' is greater than one for potassium channels of giant axons that originally created interest in single-file transport. Given that single-file transport occurs in gramicidin A channels, the finding that $n'=1$ at all sodium concentrations is further evidence that they never contain more than one sodium ion.

2. Magnitude of Single-Channel Conductance (Barriers to Ion Movement)

The maximum (small signal) sodium conductance of the gramicidin A channel in PE membranes is 14.6 pmho (*see* Fig. 5). From Eq. (8*b*) with $n'=1$ this means:

$$(M_{Na})_{[Na^+]=\infty}=2.3\times10^6 \text{ ions/sec} \tag{9}$$

where $(M_{Na})_{[Na^+]=\infty}$ is the unidirectional sodium flux through a channel (at $V=0$) at a concentration of Na^+ such that the channel always contains one sodium ion. It is instructive to compare this with $(M_{H_2O})_{[Na^+]=0}$, the unidirection water flux in an ion-free channel. This can be determined from Eq. (4*b*), in which the value of $p_d(H_2O)$ for a gramicidin A channel in PE membranes at 0.01 M NaCl is given. [At this concentration, only about 3% of the channels are occupied by a sodium ion ($K=0.31\,m$)]. Since by definition:

$$M_{H_2O}\equiv p_d(H_2O)\,c_{H_2O} \tag{10}$$

where c_{H_2O} is the concentration of water in water ($=1/18$ mol/cm^3), we have upon substituting in Eq. (4*b*):

$$(M_{H_2O})_{[Na^+]=0}=6.1\times10^7 \text{ molecules/sec.} \tag{11}$$

What would $(M_{Na})_{[Na^+]=\infty}$ be if the only impediment to Na^+ movement were the necessity to move six water molecules through the channel with the ion? This is the maximum possible value for $(M_{Na})_{[Na^+]=\infty}$. Imagine that all the Na^+ and H_2O are labeled in one compartment and none are labeled in the other—a gedanken experiment for measuring M_{Na} and M_{H_2O}. Assume also that the Na^+ concentration is such (e.g., 3 *M*) that all channels always contain one Na^+. A snapshot would reveal that, on the average, channels contain three labeled (and three unlabeled) H_2O's and 1/2 labeled (and 1/2 unlabeled) Na^+; i.e., half the channels contain a labeled Na^+ and half contain an unlabeled Na^+. Since we are assuming that Na^+ experiences the same resistance to movement as a water molecule (in an ion-free channel), but there are six times as many labeled water molecules per channel as labeled sodium ions, we must conclude that the maximum possible unidirectional sodium flux, $(M_{Na})^{m.p.}$, is given by:

$$(M_{Na})^{m.p.}=\frac{(M_{H_2O})_{[Na^+]=0}}{6} \tag{12}$$

and therefore from Eq. (11):

$$(M_{Na})^{m.p.}=1.0\times10^7 \text{ ions/sec.} \tag{13}$$

Comparing Eqs. (9) and (13) we have:

$$\frac{(M_{Na})^{m.p.}}{(M_{Na})_{[Na^+]=\infty}}=4.3. \tag{14}$$

Equation (14) is rather striking. It states that $(M_{Na})_{[Na^+]=\infty}$, the actual Na^+ flux when the channel is always occupied by sodium, is within a factor of 5 of $(M_{Na})^{m.p.}$, the maximum possible Na^+ flux, given the water permeability of an ion-free channel. This means that there is no significant electrostatic energy barrier to Na^+ movement through the channel; the rate of Na^+ movement is largely determined by the necessity for six water molecules to be moved along with the ion. In fact, Andersen and Procopio (1980) have independently determined that the translocation rate constant (l) for Na^+—i.e., the rate of Na^+ movement between the wells in Fig. 4*B*—is 2×10^7 ions/sec (in PE membranes), in complete agreement with the value in Eq. (13). [*See* Eq. (24) below for why l should be a factor of 2 larger than $(M_{Na})^{m.p.}$.] Thus, the rate of Na^+ movement within the channel (i.e., between the two energy wells at the ends) is totally determined by the rate of moving six water molecules through the channel. $(M_{Na})_{[Na^+]=\infty}$ falls short of $(M_{Na})^{m.p.}$ [*see* Eq. (14)] only because of the exit rate constant for Na^+ [k_{-1} in Eq. (24)]; i.e., because of the rate at which Na^+ can exit from the wells in Fig. 4*B*.

There is a further consequence of Eq. (14). Since $(M_{Na})^{m.p.}$ was calculated from the water permeability

of an ion-free channel, we should expect, in view of Eqs. (12) and (14), that because of the added resistance provided by the sodium ion to the movement of channel contents, $p_d(H_2O)$ in a channel containing a sodium ion would be less, by a factor of 4.3, than in an ion-free channel. This is *not* observed experimentally; instead,

$$p_d(H_2O) = 2.0 \times 10^{-15} \text{ cm}^3/\text{sec} \quad \text{(in 2 M NaCl)} \quad (15)$$

(A. Finkelstein, *unpublished results*). That is, the water permeability of a channel that always contains a sodium ion is essentially the same as that of a channel that never contains an ion [compare Eqs. (15) and (4*b*)].

This has serious implications. It can be proved (*see* Appendix A) that for a channel in which single-file movement of ions and water occurs in all parts,

$$\frac{M_{H_2O}}{M_{Na}} = N \quad (16)$$

when the channel always contains one ion. For the gramicidin A channel, Eq. (16) predicts that in 2 M NaCl

$$\frac{M_{H_2O}}{M_{Na}} \approx 6 \quad \text{(theoretical expectation in 2 M NaCl)} \quad (17)$$

whereas experimentally,

$$\frac{M_{H_2O}}{M_{Na}} = 34.9 \quad \text{(experimental result in 2 M NaCl)} \quad (18)$$

[In Eq. (18), M_{H_2O} was obtained by substituting Eq. (15) into Eq. (10), and M_{Na} was obtained by substituting 12×10^{-12} mho (the single-channel conductance in 2 M NaCl) into Eq. (8*b*).] We must therefore conclude that water can pass the sodium ion somewhere in the channel; i.e., there is not strict single filing in all parts of the channel. This same conclusion can be reached by an alternative line of reasoning (*see* Appendix B).

Let us now summarize the salient aspects of Na^+ transport through the gramicidin A channel: (1) At all attainable salt concentrations there is never more than one sodium ion in a channel. (2) There are no major electrostatic energy barriers to Na^+ transport; its rate of movement through the channel is within a factor of 5 of the maximum possible rate achievable if the only impedement to its movement was the necessity to move six water molecules in single file along with it. (3) There is not strict single-file movement of ions and water in all parts of the channel, as occupancy by a sodium ion does not depress water permeability.

The energy profile in Fig. 4*B* is consistent with the above features of sodium transport. [A similar profile (Fig. 4*A*) has been drawn by Levitt (1978), Urry et al. (1980), and Andersen and Procopio (1980); the former two, however, predict multiple-ion occupancy at high NaCl concentrations]. Two energy wells near the ends of the channel are separated by a low energy barrier (or a series of small barriers) in the middle. Strict single filing of water and ions occurs between the two wells, whereas water can pass a sodium ion sitting in either well. The most likely place for these wells is at the first turn of the β_6 helix. A sodium ion placed there can be coordinated by the carbonyl group of tryptophane 11 and by the OH group of the terminal ethanolamine; so situated, the ion allows room for water molecules to pass (*see* Fig. 3).

The absence of a significant electrostatic energy barrier for univalent cations does not imply the absence of image forces in moving an ion to the center of the channel. Apparently, however, the image forces are compensated by other electrostatic forces such as ion interactions with channel dipoles. Note that whereas the ion-dipole interaction energy is proportional to the magnitude and sign of the charge on the ion, the charging energy arising from image forces is proportional to the square of the charge on the ion, and is independent of the sign. Thus the lack of a significant electrostatic energy barrier for univalent cations does not preclude the existence of major electrostatic barriers to anion and multivalent cation movement through the channel.

D. Results with Cs^+

We turn now to the question of multiple-ion occupancy in the gramicidin A channel, using Cs^+ for illustrative purposes.

1. Multiple-Ion Occupancy

Several different lines of evidence indicate that the gramicidin A channel can be simultaneously occupied by, at least, two monovalent cations: (1) Single-channel conductance *vs.* ion activity curves are not monotonic, but pass through a maximum at high aqueous salt concentrations (Hladky and Haydon, 1972; Andersen, 1978; Neher, Sandblom & Eisenman, 1978; Urban et al., 1978). (2) At low ion concentrations, the ascending branch of the single-channel conductance *vs.* activity curve shows an inflection point (Neher et al., 1978), or a curvilinear Eadie-Hofstee plot (Eisenman, Sandblom & Neher, 1978; Neher et al., 1978; Urban et al., 1978), which indicate the existence of at least two interacting binding sites in the channel. (3) The exponent, n', of the generalized Behn-Ussing-Teorell flux-ratio equation [Eq. (8)] can

be larger than 1.0 (Schagina, Grinfeldt & Lev, 1978; Procopio & Andersen, 1979). (4) The permeability ratios, obtained by applying the Goldman-Hodgkin-Katz equation to bi-ionic potential measurements, are functions of permeant ion aqueous activities (Myers & Haydon, 1972; Eisenman et al., 1977, 1978; Urban et al., 1978, 1980). (5) "Mole-fraction-dependent" conductance behavior occurs; i.e., symmetrical replacement of a small amount of an alkali metal cation (Na^+, K^+, or Cs^+) by Ag^+ or Tl^+ produces striking decreases in the single-channel conductance, below the level observed either with the alkali metal cation itself or with complete replacement by Ag^+ or Tl^+ (Andersen, 1975, 1978; Neher, 1975; Eisenman et al., 1977; McBride & Szabo, 1978; Neher et al., 1978).

All of the above phenomena indicate interactions among ions moving through the channel; i.e., more than one ion can occupy a channel.[8] A number of plausible, albeit complex, models can account for these phenomena. For example, if one assumes that the major barrier to ion movement is the translocation step through the middle of the channel, and that therefore ion-binding sites are always in equilibrium with the aqueous phases, then a minimum of four binding sites, two at each end of the symmetrical channel, are required to account for the experimental observations (Sandblom, Eisenman & Neher, 1977). Furthermore, the rate constants for ion translocation through the channel must be functions of ion occupancy. On the other hand, if one assumes that the major barrier to ion movement is the dissociation step between the ion and the channel, and that therefore ion-binding sites are *not* in equilibrium with the aqueous phases (when there is a net flux of ions through the channel), then only two (single-filing) binding sites in the channel are required to account for the experimental observations (Hille & Schwarz, 1978; Urban & Hladky, 1979). The occupancies of the sites are in this case a function of the flux (which itself is a function of occupancies).

A choice between these two classes of models can be made from qualitative considerations. For instance, the former type of model, in which the major barrier is translocation through the channel interior, predicts that single-channel I–V characteristics should be superlinear and invariant to permeant ion activity (Hladky, Urban & Haydon, 1979). The latter type of model, in which the ion-binding sites are not in equilibrium with the aqueous phases, predicts that the shape of the I–V characteristics should be dependent upon permeant ion activity – sublinear at low ion activities, and superlinear at high ion activities. The actual behavior of gramicidin A channels is qualitatively consistent with the predictions of the latter type of model (Hladky & Haydon, 1972; Hägglund, Enos & Eisenman, 1979; Andersen & Procopio, 1980). (This model cannot account quantitatively for the shapes of the I–V characteristics at different ion activities. We believe, however, that this results from neglecting permeability limitations imposed by the aqueous convergence regions.) Thus at present, there is no compelling need to assume more than two single-filing cation-binding sites in the channel[9], and consequently in the following we interpret the Cs^+ data with the same two-site model used for Na^+.

2. Single-Channel Conductance as a Function of Permeant Ion Activity

The relationship between single-channel conductance and aqueous Cs^+ activity, c, is illustrated in Fig. 6. The conductance reaches a maximum at about 1.0 M CsCl (an acivity of 0.57 *m*) and declines with increasing ion activity at very high CsCl concentrations. Although this behavior is not consistent with the simple single-ion occupancy model presented for Na^+ and is an explicable, regular feature of models allowing multiple-ion occupancy (*see* Hille, 1979), we prefer an alternative explanation (*see* Section *D*.4.). A less striking, but equally important, deviation from the single-ion model occurs on the ascending limb of the conductance *vs.* activity curve in Fig. 6: once the conductance begins to deviate from linearity, it does not saturate as rapidly as predicted from the single-ion model of relation (6) [*see* the stippled curve in Fig. 6]. Thus, we must consider not only the occupancy of *one* of the energy minima of Fig. 4 by a Cs^+, but also the simultaneous occupancy of *both*. The channel can then exist in four different states: empty (00), left-occupied (10), right-occupied (01), and doubly-occupied (11), (*see* Fig. 7).

An analytical expression for the small-signal conductance, g, can be obtained by solving the kinetic equations associated with the graph in Fig. 7 (Heck-

[8] In section *C* we suggested an alternative explanation for the decline in Na^+ conductance at high salt concentrations; we shall propose later in section *D*.4. the same explanation for Cs^+ and other ions.

[9] There exist gramicidin A analogues, for example N-succinyl-gramicidin A, which form channels with a lower conductance than gramicidin A channels and with superlinear characteristics at all permeant ion activities (Apell, Bamberg & Alpes, 1979). These channels thus behave as if the major barrier for ion movement is indeed the translocation step through the channel interior. More detailed investigations of such analogues might help determine the number of cation-binding sites that can be simultaneously occupied in the gramicidin A channel—a question still subject to controversy (Hägglund et al., 1979; Andersen & Procopio, 1980).

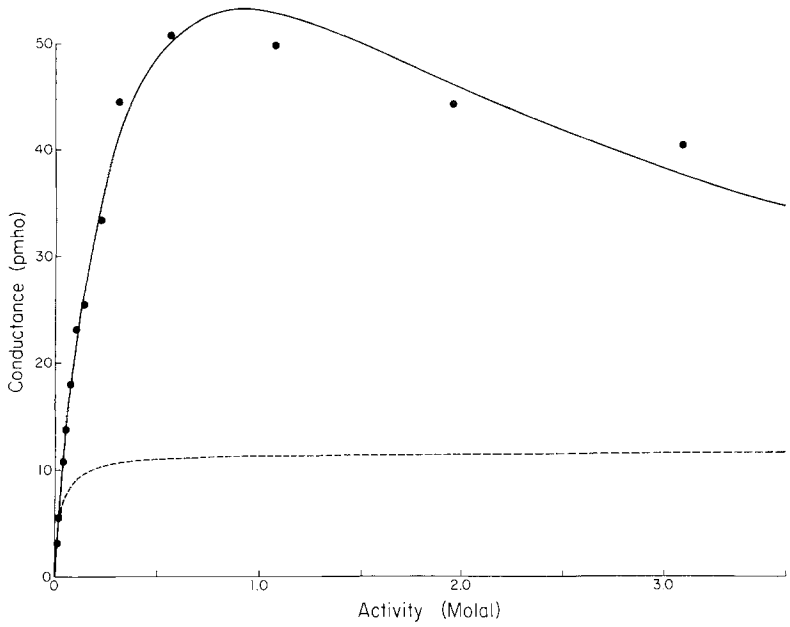

Fig. 6. Gramicin A single-channel conductance as a function of the aqueous Cs^+ activity. The solid curve is drawn according to Eq. (19) using the following parameters: $k_1 = 1.3 \times 10^8\ m^{-1}\ sec^{-1}$; $k_{-1} = 7.8 \times 10^6\ sec^{-1}$; $l = 8.9 \times 10^7\ sec^{-1}$; $k_2 = 1.4 \times 10^8\ m^{-1}\ sec^{-1}$; $k_{-2} = 5.2 \times 10^7\ sec^{-1}$; $K_1 = 0.060\ m$; $K_2 = 0.37\ m$. ($g_{max,2}$ of Eq. (25) is 70 pmho.) The stippled curve is drawn according to relation (6) with $K = 0.03\ m$, and a maximum conductance of 11.7 pmho. Diphytanoylphosphatidylcholine/*n*-decane membranes, 25 mV applied potential, $T = 25\,°C$

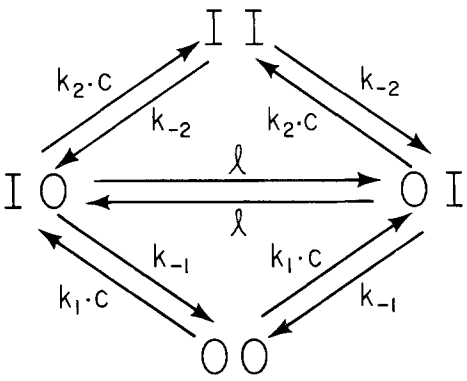

Fig. 7. Schematic illustration of the interconnections among the four kinetic states of a gramicidin A channel with double occupancy

mann, 1965; Essig, Kedem & Hill, 1966; Hille & Schwarz, 1978; Urban & Hladky, 1979):

$$g = \frac{F^2}{RT} \cdot \frac{2c}{K_1 + 2c + c^2/K_2} \cdot \frac{l(k_{-1} + k_2 c)}{2(2l + k_{-1} + k_2 c)} \qquad (19)$$

where $K_1 = k_{-1}/k_1$ and $K_2 = k_{-2}/k_2$. (Note that K in relations (6) and (7) equals $1/2K_1$, as there are two sites per channel.) The second term in Eq. (19) is the probability of finding the channel in a singly occupied state, (10) or (01), and the third term keeps track of the kinetic transitions which occur in a singly occupied channel.

The conductance of the channel (g) depends upon the probabilities of the channel being in the singly-occupied states, because in the steady state the net flux, Φ_{Cs}, can be expressed as:

$$\Phi_{Cs} = l' \cdot W(10) - l'' \cdot W(01). \qquad (20)$$

$W(10)$ and $W(01)$ are the (potential-dependent) probabilities of the channel being in states (10) and (01), respectively, and l' and l'' are the potential-dependent rate constants for translocation across the central barrier from left-to-right and right-to-left, respectively. Inspection of Fig. 7 shows that the net flux through the channel is the sum of a net flux component through the lower cycle of the diagram:

$$(00) \rightleftarrows (10) \rightleftarrows (01) \rightleftarrows (00) \qquad (21)$$

and a net flux component through the upper cycle of the diagram:

$$(01) \rightleftarrows (11) \rightleftarrows (10) \rightleftarrows (01). \qquad (22)$$

At low salt concentrations, the flux is mainly through the lower cycle, whereas at higher salt concentrations the predominant flux is through the upper; at very high salt concentrations the channel becomes "trapped" in the doubly occupied state, and conductance decreases inversely with salt activity.

One complete rotation through the lower cycle transfers an ion from one aqueous phase to the other, whereas a rotation through the upper cycle transfers an ion only if the ion in state (01) originated from the left aqueous phase – which under equilibrium conditions occurs with the conditional probability, w:

$$w = \frac{2l}{2(2l + k_{-1}) + k_2 c} \leqq 0.5 \qquad (23)$$

(O.S. Andersen, *unpublished results*). The contribution to g of the translocation process through the upper cycle may thus appear somewhat inefficient. This is not, in fact, the case; because of single-file flux-coupling effects, g is *larger* than naively estimated from the unidirectional flux [*see* Eq. (8*b*)].

Equation (19) fits the data in Fig. 6 reasonably well, but the magnitudes assigned to some of the five rate constants may be subject to revision (*see* Section D.4.). The reason that g increases above the level

predicted for a singly-occupied channel (*compare* the full and the stippled curves in Fig. 6) is that $k_{-1} < k_{-2}$, l. From Eq. (19), the maximum conductance of a singly-occupied channel ($g_{\max,1}$) is (*see also* Läuger, 1973):

$$g_{\max,1} = \frac{F^2}{RT} \cdot \frac{k_{-1} \cdot l}{2(2l + k_{-1})}. \tag{24}$$

The finding that $k_{-1} < l$ means that the conductance of the singly-occupied channel becomes limited by k_{-1}. (It also demonstrates that indeed the ion-binding sites are not in equilibrium with the aqueous phases when there is a net flux of ions through the channel.) The conductance of the doubly-occupied channel will therefore exceed the maximum value possible for the singly-occupied channel, if $k_{-2} > k_{-1}$. This inequality of the exit rate constants is in fact expected, because of repulsive interactions between the two ions in the channel.

The maximum conductance of a doubly-occupied channel ($g_{\max,2}$) is given by (O.S. Andersen, *unpublished results*)

$$g_{\max,2} = \frac{F^2}{RT} \cdot \frac{l(k_{-1} + k_{-2})}{2(2l + k_{-1} + k_{-2})}. \tag{25}$$

$g_{\max,2}$ is an upper limit; the conductance begins to decrease, at very high ion activities, before it is reached. The decrease in g_{Cs} at very high activities is described by the limiting equation:

$$g = \frac{F^2}{RT} \cdot \frac{K_2 l}{c}. \tag{26}$$

This reciprocal relation between K_2 and l at very high ion activities can be used to impose additional restrictions upon the model (as a knowledge of l serves to fix K_2 and vice versa), provided that one can be certain that the decline in g at high ion activities results from double occupancy of the channel and not from some other cause (*see* Section D.4.).

Since substantial electrostatic repulsion should exist between two cations in the channel, we expect, as noted above, that $k_{-2} > k_{-1}$. It is therefore somewhat surprising that $k_1 \approx k_2$ (*see* legend to Fig. 6), as this implies that electrostatic repulsion does not affect the height of the barrier for ion entry into the channel (*see also* Urban et al., 1980). The magnitude of k_1 (and k_2) is, however, close to that of the diffusion-controlled rate constant for ion movement up to the channel – as measured independently at very high potentials (O.S. Andersen, *manuscript in preparation*). Hence, the rate constant for crossing the barrier into the channel is not experimentally detectable, nor is the effect of any repulsive electrostatic interactions on the height of that barrier.

3. Tracer Flux Experiments (Single-File Phenomena)

a. Theoretical background. As with water, two permeability coefficients characterize the ion permeability of a membrane (Heckmann, 1972): the equilibrium permeability coefficient, P_0, and the tracer permeability coefficient, P^*. P_0 is obtained either from the small signal conductance, g, using the relation:

$$P_0 = \left(\frac{RT}{F^2} g\right) \cdot \frac{1}{c} \tag{27}$$

which can be rewritten from Eq. (19) as

$$P_0 = \left[\frac{2c}{K_1 + 2c + c^2/K_2} \cdot \frac{l(k_{-1} + k_2 c)}{2(2l + k_{-1} + k_2 c)}\right] \cdot \frac{1}{c} \tag{28}$$

or from a net flux experiment, at $V = 0$:

$$P_0 = \lim_{\Delta c \to 0} \left\{\frac{\Phi_{\text{net}}}{\Delta c}\right\} \tag{29}$$

which can be rewritten from Eq. (20) as

$$P_0 = l \cdot \lim_{\Delta c \to 0} \left\{\frac{W(10) - W(01)}{\Delta c}\right\}. \tag{30}$$

P^* is obtained from the flux of isotopically labeled ions resulting from a concentration difference of the tracer (in the absence of any applied potential):

$$P^* = \frac{\Phi^*}{\Delta c^*} \tag{31}$$

which can be rewritten from Eq. (20) as

$$P^* = l \cdot \left\{\frac{W(1^*0) - W(01^*)}{\Delta c^*}\right\} \tag{32}$$

or, with the use of Eq. (23), it becomes (O.S. Andersen, *unpublished result*)

$$P^* = \left(\frac{2c}{K_1 + 2c + c^2/K_2} \cdot \frac{l(2k_{-1} + k_2 c)}{2[2(2l + k_{-1}) + k_2 c]}\right) \cdot \frac{1}{c}. \tag{33}$$

If only a single ion can occupy the channel at any time,

$$P_0 = P^* \quad \text{(for single-ion occupancy)} \tag{34a}$$

irrespective of whether saturation phenomena exist in the channel. If, however, multiple-ion occupancy occurs,

$$P_0 \geqq P^* \quad \text{(for multiple-ion occupancy)} \tag{34b}$$

and, very importantly, the unidirectional fluxes no longer necessarily satisfy the Behn-Ussing-Teorell flux-ratio equation; that is $n' \geqq 1.0$ in Eq. (8).

It is useful to use the ratio

$$\frac{P^*}{P_0} \equiv f = \frac{1}{n'} \tag{35}$$

or from Eqs. (28) and (33)

$$f = \frac{1}{n'} = \frac{(2k_{-1}+k_2c)}{(k_{-1}+k_2c)} \cdot \frac{(2l+k_{-1}+k_2c)}{[2(2l+k_{-1})+k_2c]} \tag{36}$$

to express the degree of interaction among ions moving through the channel. The parameter f is called the correlation factor (Heckmann, 1972). Note from Eq. (36) that only three of the five rate constants enter into the expression for n' (or f) – those that describe the possible transitions in a singly-occupied channel. n' can vary between 1.0 and 2.0, but significant deviations from 1.0 occur only if $k_{-1} \leqq l$ (footnote 10). At very low ion activities, $n' = 1.0$ because of no double occupancy. At higher activities, when k_2c approaches k_{-1} and double occupancy can occur, n' may increase above 1.0 *if* $k_{-1} \leqq l$. n' continues to increase (reaching the limiting value 2.0 if $k_{-1} \ll k_2c \ll l$) until $k_2c \approx l$. At very high ion activities, when $l \ll k_2c$, n' returns to 1.0; this is associated with w in Eq. (23) approaching zero under these conditions.

b. Experimental findings. The variation of n' with Cs^+ activity is illustrated in Fig. 8. At low salt concentrations (0.01 M), $n' = 1$ ($P^* \approx P_0$), and there is little interaction among cesium ions in the channel. At higher Cs^+ concentrations, $n' > 1$ [$P^* < P_0$ or $\vec{M}/\vec{M} + \exp(-F\Delta V/RT)$], reaching a value of 1.6 at 1.0 M CsCl (0.57 m activity) – thus demonstrating multiple occupancy by Cs^+ in the gramicidin A channel. The theoretical curve was drawn according to Eq. (36) using the same estimates for the parameters k_2, k_{-1} and l (actually k_2/k_{-1} and l/k_{-1}) as were used to fit Eq. (19) to the single-channel conductances in Fig. 6.

Similar results have been obtained for Rb^+ by Schagina et al. (1978). They report that $n' = 2.0$ at both 0.01 and 0.1 M RbCl for gramicidin A channels in ox brain-lipid bilayers. The true dependence of n' on Rb^+ activity is obscured in these experiments, because the bilayers carry a negative surface charge. This causes the interfacial Rb^+ concentration to be considerably higher than in bulk solution – probably ≈ 1 M for both 0.01 M and 0.1 M RbCl concentrations. This, however, does not entirely explain the very high value of n' obtained by Schagina et al., for in diphytanoylphosphatidylcholine bilayers, n' goes through a maximum of ≈ 1.6 at 1.0 M RbCl (J. Procopio, H. Haspel and O.S. Andersen, *manuscript in preparation*). Apparently, the detailed permeability properties of the gramicidin A channel depend upon the lipid composition of the bilayer into which it is inserted.

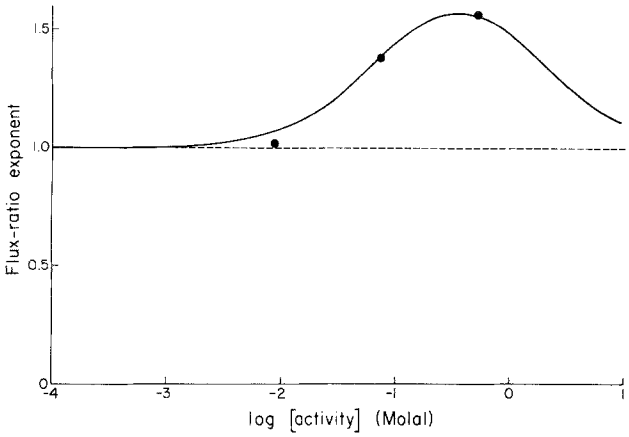

Fig. 8. Flux-ratio exponent for Cs^+ in gramicidin A channels as a function of the aqueous Cs^+ activity. Data are from Procopio and Andersen (1979) and J. Procopio, H. Haspel and O.S. Andersen (*manuscript in preparation*). The solid curve is drawn according to Eq.(36) with the same values of k_{-1}, l, and k_2 as used in Fig. 6. The stippled line is the predictions from the Behn-Ussing-Teorell flux-ratio equation, and the predictions of Eq.(36) with $l \ll k_{-1}$. Diphytanoylphosphatidyl choline/*n*-decane membranes, $T = 23$ °C

There are unfortunately no direct correlations between the magnitude of f (or n') and the number of ions in the channel. As we noted earlier with regard to Eq. (36), the state probability functions do not enter into the expression for n', only the rate constants affecting a singly-occupied channel. In fact, n' may rise measurably above 1.0 even with $c < K_1$. The variations in n' do not, therefore, give direct information about ion loading of the channel; they only show that it is possible for the gramicidin A channel to be simultaneously occupied by at least two ions (or more than two ions if $n' > 2.0$).

4. Magnitude of the Single-Channel Conductance

The rate constant for translocation through the channel (l), and its magnitude compared to other rate constants, plays a pivotal role in discussions of single-file phenomena. It is therefore of considerable

[10] This condition will almost automatically be fullfilled in single-filing channels having high conductances, because the dissociation constant for the first ion, K_1, must be low enough to ensure that a second ion can occupy the channel at accessible aqueous salt concentrations. A low value of K_1, however, necessarily means that the value of k_{-1} is low, since k_1 cannot be larger than the diffusion-limited value, $1.4 \times 10^8\, m^{-1}\, sec^{-1}$. If, for example, $K_1 = 0.05\, m$, then k_{-1} must be less than or equal to $7 \times 10^6\, sec^{-1}$.

interest that an upper estimate for its magnitude can be made by assuming that the only impediment to ion movement is the necessity for six water molecules to move through the channel each time an ion does. (Such an estimate was already given in Section *C*; the following is a more formal rendition of the same argument.) Thus, assuming k_{-1}, $k_{-2} \gg l$ (i.e., exiting from the channel is not a significant impediment to ion transport), we obtain from Eqs. (24) and (25) that

$$g_{\max,1} = g_{\max,2} = \frac{F^2}{RT} \cdot \frac{l}{2} \tag{37}$$

or, by inserting into Eq. (8b) with $n' = 1$ (from Eq. (36), $n' = 1$ when $k_{-1} \gg l$):

$$(M_{\text{ion}})^{\max} = \frac{l}{2}. \tag{38}$$

Combining with Eqs. (11) and (12), we find that

$$l = 2(M_{\text{ion}})^{\max} = \frac{(M_{H_2O})_{[\text{ion}]=0}}{3} \tag{39}$$

$$l = 2 \times 10^7 \text{ ions/sec} \tag{40}$$

which is the value independently determined for the translocation rate constant of Na^+ from electrical measurements on PE membranes (Andersen & Procopio, 1980).

The value of l in Eq. (40) is the maximum possible. Therefore, the maximum observable single-channel conductance for any ion in PE membranes should be [from Eq. (37)] less than 63 pmho. No conductances higher than this estimate are in fact observed in either PE or PC membranes. Gramicidin A channels in GMO bilayers, however, may have conductances almost two times higher than the above maximum estimate (Neher et al., 1978; Urban et al., 1980). This suggests that the water permeability $[p_d(H_2O)]$ of gramicidin A channels in GMO bilayers is at least two times higher than in PE bilayers. Movement of H_2O molecules through the channel may depend upon small, local, conformational changes of the channel wall (necessitated by the spatial constraints imposed by hydrogen-bonding of H_2O's to the polar groups in the wall), and the dynamics of such conformational changes may well depend upon the bilayer environment.

Although measured single-channel conductances in PC membranes are compatible with the magnitude of the diffusional water permeability, the estimate for l obtained by fitting Eq. (19) to the data in Figure 6 is much higher than allowed by the water permeability [Eq. (40)].[11] This estimate for l follows from the value assigned to K_2, which in turn is determined solely from the decline of small-signal conductance at high salt concentrations. We feel, however, that g_{Cs} (like g_{Na}) is lowered by large osmotic pressures, and that decreases in g_{Cs} at concentrations above 1.0 M CsCl reflect, at least in part, "nonspecific" conductance decreases associated with the large osmotic pressures at high salt concentrations. For example, g_{Cs} in 1.0 M CsCl is lowered 44% by addition of 5.0 M urea to both sides of the bilayer (O.S. Andersen, *unpublished observations*); a similar effect would be expected from large osmolarities of CsCl itself (*see* Footnote 7). Such "osmotic" effects on conductance will affect estimates of K_2 and therefore of l, as can be seen by examining Eqs. (19) and (26). Thus, in fitting Eq. (19) to small-signal conductance data such as in Fig. 6, one underestimates K_2 and thereby overestimates l (as well as other rate constants, because l is a scaling parameter, whose magnitude is determined by the need to fit both the maximum conductance *and* the conductance decrease at high salt activities).

We note that g_{Cs} is more affected by osmotic effects than g_{Na}. (5 M urea reduces g_{Cs} in 1 M CsCl by 44% but g_{Na} in 1 M NaCl by only 23%.) This is not unexpected, as the larger ion should be more sensitive than the smaller ion to stresses in the channel wall (resulting from decreases in bulk water activity), which might tend to reduce the diameter of the channel[12]. Single-channel conductance data obtained at very high salt concentrations should probably always be regarded with suspicion – whether or not they conform to preconceived notions – the more so, the larger the diameter of the permeant ion.

E. Consequences of Single-File Transport

The conductance of gramicidin A channels is affected by single-file phenomena in two quite different ways. First, there may be single filing among ions, in which case $k_{-2} > k_{-1}$, because of electrostatic repulsion between ions in the channel; consequently, channel conductance increases above the limitations imposed by k_{-1}. But secondly, even if $k_{-2} \to \infty$, channel conductance cannot increase without limit, because there is also single filing between ions and water; the rate constant for ion translocation through the channel, l,

[11] We do not believe that this discrepancy indicates the existence of more than two single-filing binding-sites, but we cannot altogether exclude this possibility. It is also possible that $p_d(H_2O)$ in PC membranes is much larger than in PE membranes, and hence l is also much larger.

[12] The channel should become longer and thinner, to minimize pressure-volume effects resulting from stresses on channel water.

is limited by the magnitude of the water permeability. As we have seen, a value of 2×10^7 ions/sec can be assigned to l for Na^+ (in PE membranes), and this value is precisely that predicted from the necessity for an ion to move six water molecules along with it when traversing the channel.

Single-file flux coupling and multiple-ion occupancy affect the permeability properties of the gramicidin A channel in other ways, not considered in this article. For example: (1) bi-ionic potentials (and permeability ratios) no longer are unique characteristic properties of the channel, as in singly-occupied channels, but depend also upon the ion-occupancy of the channel (Urban & Hladky, 1979; Hille & Schwarz, 1978) and hence upon the composition of the aqueous phases. (2) Permeability ratios no longer are exclusively determined by the heights of the energy barriers in the channel, as is the case for singly-occupied channels (Bezanilla & Armstrong, 1972; Hille, 1975*b*), but also depend upon the depths of the energy wells (Hille & Schwarz, 1978; Urban & Hladky, 1979; Kohler & Heckmann, 1980). (3) "Mole-fraction-dependent" conductance changes *may* be associated with composition-dependent permeability ratios. [Hille and Schwarz (1978) and Urban and Hladky (1979) should be consulted for further details of blocking phenomena in single-filing channels. The molecular mechanisms underlying "mole-fraction-dependent" conductances in either biological channels (Hagiwara & Takahashi, 1974; Hagiwara et al., 1977) or gramicidin A channels (Andersen, 1975, 1978; Neher, 1975; Neher et al., 1978) is still unknown, and may, at least for the gramicidin A channel, also be associated with conformational changes (Andersen & Procopio, 1980).] We have refrained from considering these issues in this article, because of complexities in evaluating both experimental data and subsequent theoretical interpretations.

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Appendix A

Theorem: Let M_{Na} and M_{H_2O} be the unidirectional fluxes of sodium and water, respectively, through a channel in which single-file transport of ions and water occurs in all parts, and let the concentration of Na^+ in the medium be such that the channel always contains one, and only one, sodium ion. Then,

$$\frac{M_{H_2O}}{M_{Na}} = N \tag{16}$$

where N is the number of water molecules in the channel.

Proof: From the formalism of irreversible thermodynamics, we write the phenomenological equations for the channel separating identical (e.g., NaCl) salt solutions (*see*, for example, de Groot, 1958):

$$I = L_{11}\Delta\Psi + L_{12}\Delta P \tag{A1a}$$

$$J_V = L_{21}\Delta\Psi + L_{22}\Delta P \tag{A1b}$$

where I is the current passing through the channel, J_V is the volume flow through it, ΔP is the hydrostatic pressure difference across it, $\Delta\Psi$ is the electrical potential difference across it, and the L's are the phenomenological coefficients. The Onsager reciprocal relations (*see* de Groot, 1958) further specify that:

$$L_{12} = L_{21} \equiv L_e. \tag{A2}$$

For the system being considered, osmotic pressure differences ($\Delta\Pi$), rather than hydrostatic pressure differences (ΔP), are applied; there is little error, however, in replacing ΔP in Eqs. (A1a) and (A1b) with $\Delta\Pi$ (*see* Levitt, Elias & Hautman (1978) for exact expressions).

The L's can be related to familiar measurable quantities. Thus, from Eqs. (A1a) and (A1b) we immediately obtain:

$$L_{11} = \left(\frac{I}{\Delta\Psi}\right)_{\Delta P=0} \equiv g_{Na} \tag{A3}$$

$$L_{12} \equiv L_e = -\left(\frac{\Delta\Psi}{\Delta\Pi}\right)_{I=0} L_{11}. \tag{A4}$$

$\left[\left(\frac{\Delta\Psi}{\Delta\Pi}\right)_{I=0}\right.$ is the streaming potential per osmolal pressure difference across the pore.$\left.\right]$

$$L_{22} = \left(\frac{J_V}{\Delta\Pi}\right)_{\Delta\Psi=0}$$

(hydrodynamic water permeability of a channel). (A5)

Substituting Eq. (1) and the definition of p_f into Eq. (A5), it becomes:

$$L_{22} = \frac{\bar{V}_w}{RT} p_f \tag{A6}$$

where $\bar{V}_w$ is the partial molar volume of water.

The quantity Q:

$$Q \equiv \frac{(L_e)^2}{L_{11} \cdot L_{22}} \tag{A7}$$

measures the degree of coupling between ions and water and can take on values between 0 and 1 (Lorenz, 1952). When it is near 0, there is little coupling; this is expected at low salt concentrations (e.g., 0.01 M NaCl for the gramicidin A channel), where the channel rarely contains an ion and therefore most water flow is not coupled to ion movement. When Q is 1, on the other hand, there is perfect coupling of ion and water movement. This is expected at very high NaCl concentrations, where a channel almost always contains an ion and therefore, because of the single-file nature of transport, water and ion flows are inextricably coupled.

Substituting Eq. (A4) into Eq. (A7), we have:

$$Q = \frac{L_{11}}{L_{22}} \left(\frac{\Delta\Psi}{\Delta\Pi}\right)^2_{I=0} \tag{A8}$$

and since $(\Delta\Psi/\Delta\Pi)_{I=0} = N\bar{V}_w/F$ [*see* Eq. (5) of Finkelstein and Rosenberg (1979)], this becomes:

$$Q = \frac{L_{11}}{L_{22}} \cdot N^2 \left(\frac{\bar{V}_w}{F}\right)^2. \tag{A9}$$

Under the conditions of the theorem, there is perfect coupling of ion and water flow; therefore $Q = 1$ and Eq. (A9) becomes:

$$\frac{L_{11}}{L_{22}}=\left(\frac{F}{N\bar{V}_w}\right)^2. \tag{A10}$$

Substituting Eqs. (A3) and (A6) into this we have:

$$\frac{g_{Na}}{p_f}=\frac{F^2}{RT\bar{V}_wN^2} \tag{A11}$$

But we also have from Eq. (8b) that

$$M_{Na}=\frac{RT}{F^2}g_{Na} \tag{A12}$$

and substituting this into Eq. (A11) we get:

$$\frac{M_{Na}}{p_f}=\frac{1}{N^2\bar{V}_w}. \tag{A13}$$

But,

$$\frac{p_f}{p_d(H_2O)}=N \tag{3}$$

so that,

$$\frac{M_{Na}}{p_d(H_2O)}=\frac{1}{N\bar{V}_w}. \tag{A14}$$

But also,

$$M_{H_2O}=p_d(H_2O)c_{H_2O} \tag{10}$$

and substituting this into Eq. (A14) we finally obtain:

$$\frac{M_{H_2O}}{M_{Na}}=c_{H_2O}\bar{V}_wN=N. \quad \text{Q.E.D.}$$

Appendix B. Alternative Demonstration That There is Not Strict Single Filing in All Parts of the Gramicidin A Channel

If there were strict single filing of ions and water, then at 5 M NaCl, where all channels contain one sodium ion, Q of Eq. (A7) should be 1. Data do not exist at 5 M NaCl for all of the L's of Eq. (A7) to test this prediction, but they do exist at 1 M NaCl.

What do we expect Q to be in 1 M NaCl? At this concentration $g_{Na}=9.2\times10^{-12}$ mho, a value 63% that of the maximum conductance (14.6×10^{-12} mho). A channel is therefore occupied by a sodium ion 63% of the time and is ion-free 37% of the time[13]. Since M_{H_2O} for an ion-free channel is 4.3 times larger than predicted from M_{Na} in an ion-occupied channel [*see* Eqs. (12) and (14)], L_{22} for an ion-free channel is 4.3 times larger than its predicted value in an ion-occupied channel. Therefore, from Eq. (A7):

$$Q^{(\text{theoret})}_{1\text{M NaCl}}=\frac{(L_e)^2}{L_{11}\left[0.63\dfrac{(L_e)^2}{L_{11}}+0.37\times4.3\dfrac{(L_e)^2}{L_{11}}\right]}=0.45. \tag{B1}$$

Experimentally, however, we find that:

$$Q^{(\text{exp})}_{1\text{M NaCl}}=0.11. \tag{B2}$$

[13] From the value of 0.31 m for K in Eq. (7), the channel is occupied by a sodium ion 76% of the time. (g_{Na} in 1 M NaCl is slightly lower than predicted by the best-fit curve in Fig. 5.) If we use this in our calculation, $Q^{(\text{theoret})}$ in Eq. (B1) is 0.56 instead of 0.45.

(This is calculated from Eq. (A7) with $L_{11}=9.2\times10^{-12}$ mho [Eq. (A3)], $L_{12}=0.86\times10^{-14}$ mho cm^3 C^{-1} [Eq. (A4) with $(\Delta\Psi/\Delta\Pi_{I=0}=2.35\times10^{-3}$ V osmolal^{-1}], and $L_{22}=7.6\times10^{-17}$ cm^6 V^{-1} C^{-1} sec^{-1} [Eq. (A6) with $p_f=10.6\times10^{-15}$ cm^3/sec; this last value is obtained by assuming that $p_d(H_2O)$ *in* 1 M NaCl *is* the same as in 2 M NaCl and that $p_f/p_d=5.3$].) Thus Q is about a factor of 4 smaller in 1 M NaCl than predicted from strict single-file transport of ions and water. A similar discrepancy is found between the actual and predicted values of M_{H_2O}/M_{Na} in 2 M NaCl [*compare* Eqs. (18) and (17)].

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