J. Membrane Biol. 73, 203–216 (1983)

The Journal of
Membrane Biology

Concentration Dependence of Bidirectional Flux Ratio as a Characteristic of Transmembrane Ion Transporting Mechanism

L.V. Schagina, A.E. Grinfeldt, and A.A. Lev
Laboratory of Physical Chemistry of Cell Membranes, Institute of Cytology of the Academy of Sciences of the USSR, Leningrad

Summary. Unidirectional flux ratio for monovalent cations was determined by tracer flux and conductivity measurements on lipid bilayer membranes formed from bulk ox brain lipids modified by valinomycin, gramicidin A and O-pyromellitylgramicidin A. Deviations from unity of the flux ratio index n in Ussing's equation were regarded as evidence for nonindependent ionic movement across the membranes. Valinomycin-modified membranes in RbCl solutions showed the index n close to unity for the salt concentrations up to 5×10^{-2} M while for the membranes in 10^{-1} M RbCl it decreased to 0.54 ± 0.16, indicating a significant contribution of exchange diffusion. Gramicidin A-treated membranes in 10^{-1} M chlorides showed the index n greater than unity: 1.21 ± 0.15, 1.68 ± 0.15 and 1.99 ± 0.21 for Na^+, Cs^+ and Rb^+ fluxes, respectively. The index n exceeding the unity was considered as a manifestation of bidirectional flux interaction in the single-file pore. Dependence of the index n on RbCl concentration showed obvious maximum at 10^{-2} to 10^{-1} M solutions ($n\simeq2$). Decrease in the index n values down to 1.47 ± 0.15 at 1.0 M RbCl evidenced in favor of a two-site model of a gramicidin A channel. An introduction of negative charges to the entrance of channels when formed by O-pyromellitylgramicidin did not alter the index n value, the fact expected if ion binding sites are located near membrane interface.

Key Words ionic flux interactions · tracer studies · bilayers · gramicidin · valinomycin

Introduction

A. Hodgkin and R. Keynes (1955) were the first to experimentally test the independence principle of ionic movement across cell membranes. As a criterion of independent ionic movement through membrane the authors used the applicability of the relation of unidirectional ionic flux ratio to driving forces known in biology as the Ussing equation (Behn, 1897; Teorell, 1935; Ussing, 1949; Hodgkin & Huxley, 1952):

$$\vec{\phi}_i/\overleftarrow{\phi}_i=\frac{C_i'}{C_i''}\exp(z_iVF/RT) \tag{1}$$

where C_i' and C_i'' are the concentrations of the ion i of the valence z_i in the outer and inner compartments separated by the membrane, V is the potential difference at the membrane, R, F and T are the gas constant, the Faraday number and the absolute temperature, respectively. Studies on the influx and efflux of potassium ions in the poisoned giant axon of *Sepia* officinalis as well as unidirectional flux ratio as a function of driving forces showed that the movement of potassium ions is not independent and that the interaction of bidirectional fluxes might be represented by a correctional exponent n when introduced to Eq. (1):

$$\vec{\phi}_i/\overleftarrow{\phi}_i=\left[\frac{C_i'}{C_i''}\exp(z_iVF/RT)\right]^n . \tag{2}$$

The mean value of the index n for the potassium transporting system in the *Sepia* giant axon membrane was found equal to 2.5. Later on, a number of determinations of unidirectional flux ratio for potassium-conducting channels (delayed rectifiers) of nerve membranes (Begenisich & DeWeer, 1977, 1980) and a functionally similar system of inward rectifiers of muscle cells (Horowicz, Gage & Eisenberg, 1968) and echinoderm egg membranes (Hagiwara, Miyazaki & Rosenthal, 1976; Hagiwara & Yoshii, 1979) proved the existence of an interaction of bidirectional ionic fluxes. Experimental finding of a strong influence of the ionic composition of the outer and inner media on the ionic permeability coefficients and a prominent conductance blocking in the presence of some permeating ions in the media as well as saturation in current-voltage characteristics show the important role of ionic flux interactions in the potassium transporting system of cell membranes. The increasing number of data on the dependence of conductivity and ionic selectivity of sodium and calcium channels of electrically excitable cell membranes from the ionic composition of the media suggests that the independence principle might also be inapplicable to the ionic movement in these channels (Chandler, Hodgkin & Meves, 1965; Chandler & Meves, 1965; Meves & Vogel, 1973; Hille, 1975*a*, *b*; Cahalan & Begenisich, 1976; Kostyuk & Krishtal, 1977).

An urgent request for a deeper understanding of the interaction mechanism of ionic fluxes in the above physiologically important transporting systems stimulated a search for an adequate model allowing a detailed physico-chemical study of the phenomenon.

A pore which contains several permeating ions and is so narrow that the ions are not permitted to pass each other (single-file diffusion mechanism) might be one of the simplest but not the only possible theoretical model accounting for bidirectional flux interactions. The theory of single-file diffusion developed first by A.L. Hodgkin in 1952 (*personal communication*) and Hodgkin and Keynes (1955) assumed that the value of the index n in Eq. (2) for a given extra narrow pore should be dependent on the number of ions in a single file and the number of vacant sites for ions in the pore. The further development of the theory of single-file diffusion in a series of detailed studies by K. Heckmann and coworkers (Heckmann, 1965*a*, *b*; 1968, 1972; Heckmann, Vollmerhaus, Kutschera & Vollmerhaus, 1969; Heckmann & Vollmerhaus, 1970; Kohler & Heckmann, 1979, 1980) demonstrated that the quantitative meaning of the index n as a parameter determining the number of particles in the pore critically depended on the number of vacancies there. Experimental support of the theory already used for characterizing the properties of channels both in biological and artificial membranes (Eisenman, Sandblom & Neher, 1977, 1978; Sandblom, Eisenman & Neher, 1977; Hille & Schwarz, 1978; Hladky, Urban & Haydon, 1979) seems to be possible when the applicability of the theory is tested on channels of known number of ions sitting inside the pore. Changes in the number of vacancies in the pore achieved by different pore loading which varied with the concentration of ions in adjacent solutions might be regarded as another possibility to check the theory. The mode of the index n changing with the concentration of permeating ions in the solution has been theoretically predicted in the recent paper by Kohler and Heckmann (1980) for the most important cases of single-file pore containing from two to five particles.

Despite a number of investigations illustrating the major role of interactions of ionic fluxes in biological and artificial membranes there exist only a few publications concerned with direct determinations of unidirectional ionic flux ratios for membranes. No special experimental studies have been made on concentration dependence of index n. Limitations of ionic compositions of the media obvious for biological membranes might be much diminished in the case of artificial lipid bilayers modified by channel-forming compounds. Among them, gramicidin A, a neutral linear pentadecapeptide of known primary structure (Urry, Goodall, Glickson & Mayers, 1971) has been most thoroughly studied. Dimers of head-to-head (formil to formil ends) connected gramicidin A molecules in π^6(LD)-helical conformation stabilized by intra- and intermolecular hydrogen bonds form in lipid bilayer channels of about 26 Å long with an effective inner pore diameter of about 4 Å (Urry et al., 1971; Bamberg & Janko, 1977; Weinstein, Wallace, Blout, Morrow & Veatch, 1979). The results of detailed studies on the electrochemical properties of gramicidin A-treated membranes obtained during the last decade are in complete accordance with the phenomena predicted for extra narrow pore formed in the membranes by the antibiotic (for a review *see* Bamberg et al., 1978; Eisenman et al., 1978). When the above-mentioned findings of electrochemical investigations are interpreted in terms of the single-file diffusion theory two particular cases of the model for gramicidin A channels spanning bilayer membranes with two or four binding sites may be suggested as the most probable. Experimental evidence in favor of the models has been discussed by several groups of investigators (Sandblom et al., 1977; Eisenman et al., 1978; Levitt, 1978*a, b;* Hladky et al., 1979; Urry et al., 1980; *see also* Bamberg et al., 1978; Hille & Schwarz, 1978; Bamberg et al., 1979). Some of the papers cited emphasized the importance of determining the unidirectional flux ratio in order to discriminate between the two most probable models of the channel. Preliminary data on the index n for gramicidin-treated membranes (Andersen & Procopio, 1978, 1980; Schagina, Grinfeldt & Lev, 1978, 1980) are not sufficient to make a final choice because of uncertain occupancy of channel sites under given experimental ionic conditions. As has already been mentioned the concentration dependence of the index n is a better approach to the problem. In this paper we report on data concerning the index n for gramicidin A-treated membranes studied in a wide range of RbCl solutions and compare the results with the concentrations dependence of the index n for valinomycin dopped bilayers, which represent different ion-transporting mechanisms. It was of interest also to correlate values of the index n obtained for gramicidin A-treated bilayers in the presence of other cations (Cs^+ and Na^+) and for Rb^+ ions passing channels formed in the bilayers by a negative charged derivate – O-pyromellityl-gramicidin A.

Materials and Methods

Spherical lipid bilayer membranes were formed at the cut off and thoroughly polished end of a thick wall glass capillary (inner diameter 0.8, outer 8 mm) by the method similar to that described by Kübel (1975; *see also* Jung, 1971). About 0.5 to 0.8 ml of RbCl, CsCl or NaCl solutions containing ^{86}Rb, ^{137}Cs, ^{22}Na, respectively (in some experiments also ^{36}Cl in NaCl solution), as the tracers with specific radioactivity of about 50 $\mu Ci\ ml^{-1}$ were sucked in with the aid of a syringe inside the capillary until the solution made contact with the wire Ag–AgCl inner electrode. After that the end of the capillary was thoroughly wiped out several times with wet filter paper and 0.01 to 0.03 ml of membrane-forming lipid solution was sucked in. The latter solution contained 1.5% of bulk ox brain lipids together with cholesterol added in 1:1 (wt/wt) ratio and dissolved in 4:3:2 (vol/vol) chloroform/tetradecane/methanol mixture. Then the capillary was immersed into a rectangular quartz cuvette with 10 ml of the same electrolyte solution as that inside the capillary but initially containing no tracer. The second wire Ag–AgCl electrode was brought in contact with the outside solution. The bubble of the thick (color) membrane was blown up by an accurate shift of plunger of a syringe connected to the capillary. After 30 to 60 min of intensive stirring of the outside solution the process of bubble wall thinning was completed and the black bilayer membrane of an area up to 2.5 cm^2 was thus formed. In successful experiments bubbles of the bilayer membranes stayed for 30 to 70 h. The membrane surface area was calculated from the diameter and the height of spherical segment measured with the aid of the stereoscopic microscope. The membrane surface area was kept constant in the course of the experiments with an accuracy better than $\pm 2\%$ when displacements of the level of the inside solution in the capillary were compensated by very delicate shifts of the syringe plunger.

Unbuffered solutions (pH $\simeq$ 6) of Rb, Cs and Na chlorides of extra purity brand in distilled water were used in experiments. Antibiotics dissolved in methanol were added to the outer solutions. Final concentration of the alcohol in electrolyte solutions never exceeded 1 vol/100 vol which by itself did not influence notably the permeability of the membranes. In experiments we used commercial gramicidin mixture ("Sigma") which contains 70 to 85% of gramicidin A, 5 to 10% of gramicidin B and 7 to 20% of gramicidin C. Experimental results obtained with this preparation were compared and found similar to those for gramicidin A purified according to the procedure described by Gross and Witkop (1965). The latter preparation was a generous gift of Professor V.T. Ivanov (Institute of Bioorganic Chemistry of the Academy of Sciences of the USSR). O-pyromellitylgramicidin A was most kindly presented by Professor P. Läuger (University of Konstanz, Germany). Valinomycin was obtained from commercial suppliers, primarily "Calbiochem." In the course of the experiments electrolyte solutions were thermostated at 27 ± 1 °C.

The tracer determined efflux from the bubble (ϕ_i^*) was calculated from the rate of tracer gain by the outer solution:

$$\vec{\phi}_i^* = \frac{\Delta N_i^* \cdot C_i}{\Delta t \cdot S \cdot C_i^*}, \tag{3}$$

where ΔN_i^* is the increase of activity of the tracer in the outer solution reached at the time interval Δt, C_i and C_i^* are the concentrations of cold and radioactive isotopes, respectively, in the inner solution and S is the membrane area. The efflux thus determined needed no correction for the tracer influx since the tracer concentration of the outer solution never exceeded 0.01% of that inside the bubble. Samples of the outer solutions were taken over 20 to 30-min intervals. The weight of each sample (0.2 or 0.4 g) was defined with a precision of ± 0.5 mg after the transfer of the sample to a previously weighed tube with a scintillator. β-radioactivity of the samples was determined by liquid scintillation counters "Mark II" or "Beckman." Commercially available ("Isotope") preparations of $^{86}RbCl$, $^{137}CsCl$, $^{22}NaCl$ and $Na^{36}Cl$ were used in experiments after additional tests for radiochemical purity of the preparations by high-resolution Ge(Li) γ-spectrometer.

Fairly high concentrations of the gramicidin A in water salt solutions should be used in experiments to get rates of tracer gain higher than 3 counts per min^2 permitting to determine the ionic fluxes with a precision better than $\pm 10\%$. In the course of the work it was found that the constancy of bulk concentration of gramicidin A in the system was not sufficient to have constant tracer-measured fluxes and membrane conductance during the whole time of prolonged experiments. Even at concentrations of the antibiotic in solutions exceeding saturation (easily detectable by the appearance of small flakes of the substance) there was a steady decline in tracer flux and membrane conductance with time. Different membranes including those made from the same membrane-forming solution behaved differently in respect to this steady decline of the membrane permeability. That is why it was found reasonable to keep constant not the bulk concentration of gramicidin A in solutions but the conductance of membranes. The latter experimental condition was fulfilled by addition of small portions of freshly prepared, concentrated gramicidin A solutions in the same electrolyte as that outside the bubble to restore the membrane conductance.

The specific membrane conductance at zero current condition ($G_o = 1/S(\partial I/\partial V)_{V \to 0}$) was determined for each 20 to 30-min interval by recording transmembrane current (I) when controlled potential differences (V) up to ± 15 mV were applied to obtain several points of the current-voltage characteristics of the membrane. These characteristics were found perfectly linear for $V < 50$ mV.

Only the efflux $\vec{\phi}_i^*$ could be measured by the experimental set employed in this work. However for symmetrical electrolyte systems used throughout the experiments, change in the sign of the potential difference across the membrane was equivalent to the transfer of the efflux measurement to the measurement of the influx at a given potential difference, i.e. $\overleftarrow{\phi}_V^* = \vec{\phi}_{-V}^*$. (It is obvious that for $V = 0$, $\overleftarrow{\phi}_i^* = \vec{\phi}_i^*$). Thus the ratio of unidirectional fluxes of an ion $\vec{\phi}_i^*/\overleftarrow{\phi}_i^*$ at a potential difference V may be determined as the ratio $\vec{\phi}_V^*/\vec{\phi}_{-V}^*$. Correspondently the index n may be found as:

$$n = \frac{[\ln(\vec{\phi}_i^*/\overleftarrow{\phi}_i^*)]RT}{z_i FV} = \frac{[\ln(\vec{\phi}_V^*/\vec{\phi}_{-V}^*)]RT}{z_i FV}. \tag{4}$$

Following Hodgkin and Keynes (1955), as the second independent method for determination of the index n we used the relation between membrane conductance G_o and tracer-measured efflux $\vec{\phi}_i^*$ at zero current condition:

$$n = \frac{t_+ G_o RT}{\vec{\phi}_i^* z_i^2 F^2} \equiv \frac{P_i}{P_i^*}, \tag{5}$$

where t_+ is the transference number of the cation i present in the solution. Knowing t_+ it is enough to measure simultaneously G_o and $\vec{\phi}_i^*$ for determination of the index n. The relation (5) is equivalent to ratio P_i/P_i^* if one defines a tracer-determined permeability coefficient of the membrane as $P_i^* = \vec{\phi}_i^*/C_i$ and an electrically measured permeability coefficient for the same membrane as $P_i = t_+ G_o RT/z_i^2 F^2 C_i$.

The third method for determining the index n was based on simultaneous measurements of the cation net flux (ϕ_V) and a tracer-determined efflux ($\vec{\phi}_i^*$) at a given potential difference (V or $-V$) across the membrane. A net flux of the cation

i was found as $\phi_V = G_o t_+ SV/z_i F$. Knowing the cation net flux ϕ_V and tracer-determined efflux the influx of the cation might be found: $\overleftarrow{\phi}_i = \vec{\phi}_i^* - \phi_V$. As has already been noted the change of the sign of potential difference across the membrane from V to $-V$ was equivalent to transition from the measurement of $\vec{\phi}_i^*$ to that of $\overleftarrow{\phi}_i^*$. Then the index n might be determined from the tracer-measured efflux at V or $-V$ and the membrane conductance found under the same conditions:

$$n = \frac{RT}{Vz_iF} \ln\left(\frac{\vec{\phi}_i^*}{\vec{\phi}_i^* - \phi_V}\right) \tag{6a}$$

$$n = \frac{RT}{Vz_iF} \ln\left(\frac{\phi_V + \overleftarrow{\phi}_i^*}{\overleftarrow{\phi}_i^*}\right). \tag{6b}$$

Cation transference numbers necessary for finding the index n with the aid of the last two methods were obtained either in a special series of experiments from the slope of a linear dependence of potential difference across membranes modified by antibiotics on the log of a salt activity varied at the outer solutions or for the same membranes as used in unidirectional flux ratio determinations by comparison of a tracer-measured cation net flux ($\vec{\phi}_i^* - \overleftarrow{\phi}_i^* = \vec{\phi}_V^* - \vec{\phi}_{-V}^*$) and transmembrane current I at potential difference V and $-V$:

$$t_+ = \frac{(\vec{\phi}_i^* - \overleftarrow{\phi}_i^*) z_i F}{I} = \frac{(\vec{\phi}_V^* - \vec{\phi}_{-V}^*) z_i F}{I}. \tag{7}$$

Results

From a series of publications (Myers & Haydon, 1972; Urban, Hladky & Haydon, 1980) it is known that the anion permeability of gramicidin A channels is negligible. But some indirect evidence obtained by G. Eisenman and coworkers (Sandblom et al., 1977; Eisenman et al., 1978) indicates that in salt solutions with concentrations higher than 10^{-1} M the anion permeation through gramicidin A channels should be taken into account (except for SO_4^{2-}). All data on cation versus anion selectivity available have been obtained on bilayers prepared of lipids different from those used in this work. That compelled us to determine the cation transference numbers under the same conditions and on the same bilayers as those used for defining bidirectional flux interactions. Data on the transference numbers for cations obtained by the comparison of tracer-determined cation net fluxes and electrical current at a given potential difference are presented in Table 1. Special series of experiments on potentiometric determinations of t_+ were made for gramicidin A-treated membranes in RbCl solutions with concentrations close to 1.0 M. The slope of the linear dependence of transmembrane potential differences on the log of concentrations of the solution varied at one side of the membrane gave t_+ values between 0.8 and 0.9. But when streaming potentials that arise at unequal outer and inner electrolyte concentrations were minimized by compensation of a difference in the osmotic pressure of the solutions by addition of appropriate amounts of urea (for details *see* Rosenberg & Finkelstein, 1978) the value of t_+ increased up to 0.95. The difference between the latter value and the unity was within limits of experimental error. Taking into account similar results concerning the change of t_+ with osmotic corrections obtained by Rosenberg and Finkelstein (1978) we came to the conclusion that for all concentrations of chlorides used in this work 1.0 molar including t_+ might be considered equal to unity. Chlorine flux measurements performed with the aid of ^{36}Cl proved that gramicidin A-treated membranes are several orders less permeable for the anion compared with Na^+.

Data on the index n values obtained for gramicidin A-treated membranes in RbCl solutions of different concentrations presented in Table 2 allowed the following conclusions:

1. The fact that the index n values significantly exceed the unity is obvious in the case of gramicidin A channels transporting Rb^+ ions since not in a single individual membrane and at no concentrations of RbCl was n found to be less than 1.3.

Table 1. Tracer-determined cation transference numbers for gramicidin A and O-pyromellitylgramicidin A-modified bilayers[a]

Salt	Salt concentration (M)	Specific conductivity ($S \cdot cm^{-2}$)	Potential applied (mV)	Transference number t_+
A. Gramicidin A				
RbCl	1.0	$4.7 \cdot 10^{-6}$	±29	1.0
	10^{-1}	$9.8 \cdot 10^{-7}$	42	1.0
	10^{-1}	$1.2 \cdot 10^{-6}$	41	0.9
	10^{-1}	$1.9 \cdot 10^{-6}$	67	1.0
	10^{-1}	$2.6 \cdot 10^{-6}$	±29	1.0
	10^{-1}	$2.8 \cdot 10^{-6}$	67	1.1
	10^{-1}	$3.7 \cdot 10^{-6}$	81	0.9
	10^{-1}	$7.2 \cdot 10^{-6}$	±31	1.0
	10^{-2}	$2.3 \cdot 10^{-7}$	±22	0.9
	10^{-2}	$6.0 \cdot 10^{-7}$	±18	0.9
	$3 \cdot 10^{-3}$	$1.4 \cdot 10^{-8}$	35	1.0
			mean and SD	1.00±0.07
CsCl	10^{-1}	$6.6 \cdot 10^{-6}$	±24	1.0
NaCl	10^{-1}	$2.5 \cdot 10^{-7}$	±24	1.2
	10^{-1}	$1.9 \cdot 10^{-6}$	±29	0.9
B. O-pyromellitylgramicidin A				
RbCl	10^{-1}	$2.5 \cdot 10^{-7}$	116	1.0
	10^{-1}	$8.6 \cdot 10^{-6}$	±20	0.9

[a] Here and in all Tables below potential difference signified by $-V$ corresponds to the electric field preventing the efflux of cations.

Table 2. Experimental conditions tracer-determined cation efflux and the index n for gramicidin A-treated lipid bilayers in RbCl solutions

Salt concentration (M)	Membrane area (cm^2)	Potential applied (mV)	Specific conductivity ($S \cdot cm^{-2}$)	Tracer-determined efflux ($mol \cdot cm^{-2} \cdot s^{-1}$)	n	n_{cor}
$2.2 \cdot 10^{-3}$	1.8	0	$3.7 \cdot 10^{-8}$	$5.68 \cdot 10^{-15}$	1.7	1.9
$2.2 \cdot 10^{-3}$	1.8	0	$4.0 \cdot 10^{-8}$	$6.51 \cdot 10^{-15}$	1.6	1.7
$2.2 \cdot 10^{-3}$	1.8	0	$4.6 \cdot 10^{-8}$	$7.92 \cdot 10^{-15}$	1.5	1.6
$2.2 \cdot 10^{-3}$	1.8	0	$5.1 \cdot 10^{-8}$	$8.65 \cdot 10^{-15}$	1.6	1.6
$2.2 \cdot 10^{-3}$	1.8	0	$8.9 \cdot 10^{-8}$	$1.21 \cdot 10^{-14}$	1.9	2.0
$2.2 \cdot 10^{-3}$	1.6	0	$1.3 \cdot 10^{-7}$	$2.00 \cdot 10^{-14}$	1.7	1.8
$2.2 \cdot 10^{-3}$	1.6	10	$5.2 \cdot 10^{-8}$	$1.25 \cdot 10^{-14}$	1.5	1.5
$2.2 \cdot 10^{-3}$	1.6	6	$8.7 \cdot 10^{-8}$	$1.65 \cdot 10^{-14}$	1.7	1.7
$2.2 \cdot 10^{-3}$	1.6	−33	$1.9 \cdot 10^{-7}$	$9.52 \cdot 10^{-15}$	1.6	1.6
				mean and SD	1.64 ± 0.12	1.71 ± 0.16
10^{-2}	1.7	0	$1.0 \cdot 10^{-7}$	$1.26 \cdot 10^{-14}$	2.2	2.4
10^{-2}	1.7	0	$2.5 \cdot 10^{-7}$	$3.01 \cdot 10^{-14}$	2.2	2.3
10^{-2}	1.7	0	$7.6 \cdot 10^{-7}$	$9.40 \cdot 10^{-14}$	2.1	2.2
10^{-2}	1.7	0	$9.7 \cdot 10^{-7}$	$1.49 \cdot 10^{-13}$	1.7	1.7
10^{-2}	1.7	22 −22	$2.3 \cdot 10^{-7}$	$5.74 \cdot 10^{-14}$ $1.12 \cdot 10^{-14}$	1.9	2.0
10^{-2}	1.7	−17	$2.5 \cdot 10^{-7}$	$1.45 \cdot 10^{-14}$	2.1	2.2
10^{-2}	1.7	−14	$4.3 \cdot 10^{-7}$	$3.05 \cdot 10^{-14}$	2.0	2.1
10^{-2}	1.7	−19	$5.2 \cdot 10^{-7}$	$2.70 \cdot 10^{-14}$	2.1	2.2
10^{-2}	1.7	18 −18	$6.0 \cdot 10^{-7}$	$1.32 \cdot 10^{-13}$ $3.24 \cdot 10^{-14}$	2.0	2.0
10^{-2}	1.7	−17	$7.0 \cdot 10^{-7}$	$1.67 \cdot 10^{-13}$	2.1	2.1
				mean and SD	2.04 ± 0.15	2.12 ± 0.19
10^{-1}	1.5	0	$1.8 \cdot 10^{-6}$	$2.22 \cdot 10^{-13}$	2.1	2.4
10^{-1}	1.6	0	$1.8 \cdot 10^{-6}$	$2.90 \cdot 10^{-13}$	1.6	1.8
10^{-1}	1.5	0	$2.2 \cdot 10^{-6}$	$2.97 \cdot 10^{-13}$	2.0	2.1
10^{-1}	1.3	0	$2.2 \cdot 10^{-6}$	$2.81 \cdot 10^{-13}$	2.1	2.3
10^{-1}	1.3	0	$3.0 \cdot 10^{-6}$	$3.73 \cdot 10^{-13}$	2.1	2.2
10^{-1}	1.3	0	$6.2 \cdot 10^{-6}$	$7.80 \cdot 10^{-13}$	2.1	2.2
10^{-1}	1.3	0	$6.3 \cdot 10^{-6}$	$7.67 \cdot 10^{-13}$	2.2	2.2
10^{-1}	1.3	0	$9.9 \cdot 10^{-6}$	$1.44 \cdot 10^{-12}$	1.8	1.8
10^{-2}	1.3	0	$1.6 \cdot 10^{-5}$	$2.53 \cdot 10^{-12}$	1.7	1.7
10^{-1}	1.5	−30	$1.6 \cdot 10^{-6}$	$7.08 \cdot 10^{-14}$	1.8	2.1
10^{-1}	1.6	−15	$1.8 \cdot 10^{-6}$	$1.26 \cdot 10^{-13}$	2.0	2.2
10^{-1}	1.3	32	$2.4 \cdot 10^{-6}$	$9.26 \cdot 10^{-13}$	1.7	1.9
10^{-1}	1.3	29 −29	$2.6 \cdot 10^{-6}$	$8.92 \cdot 10^{-13}$ $8.04 \cdot 10^{-14}$	2.1	2.4
10^{-1}	1.3	−26	$2.8 \cdot 10^{-6}$	$9.82 \cdot 10^{-14}$	2.1	2.3
10^{-1}	1.3	31	$6.9 \cdot 10^{-6}$	$2.51 \cdot 10^{-12}$	1.9	1.9
10^{-1}	1.3	31 −31	$7.2 \cdot 10^{-6}$	$2.55 \cdot 10^{-12}$ $1.67 \cdot 10^{-13}$	2.3	2.4
10^{-1}	1.3	−30	$7.6 \cdot 10^{-6}$	$1.80 \cdot 10^{-13}$	2.3	2.4
				mean and SD	1.99 ± 0.21	2.14 ± 0.23
1.0	1.4	0	$4.3 \cdot 10^{-6}$	$6.73 \cdot 10^{-13}$	1.7	2.2
1.0	1.8	0	$4.8 \cdot 10^{-6}$	$9.77 \cdot 10^{-13}$	1.3	1.6
1.0	1.8	0	$7.7 \cdot 10^{-6}$	$1.33 \cdot 10^{-12}$	1.5	1.7
1.0	0.7	0	$8.2 \cdot 10^{-5}$	$1.55 \cdot 10^{-11}$	1.4	1.4
1.0	1.4	29 −29	$4.7 \cdot 10^{-6}$	$1.81 \cdot 10^{-12}$ $4.05 \cdot 10^{-13}$	1.3	1.7
1.0	1.8	18	$5.1 \cdot 10^{-6}$	$1.55 \cdot 10^{-12}$	1.4	1.6
1.0	1.8	17	$6.8 \cdot 10^{-6}$	$1.83 \cdot 10^{-12}$	1.6	1.8
1.0	1.4	−29	$5.7 \cdot 10^{-5}$	$4.80 \cdot 10^{-12}$	1.4	1.4
1.0	0.7	16	$9.0 \cdot 10^{-5}$	$2.67 \cdot 10^{-11}$	1.4	1.4
1.0	0.7	13	$1.3 \cdot 10^{-4}$	$3.07 \cdot 10^{-11}$	1.7	1.7
				mean and SD	1.47 ± 0.15	1.65 ± 0.24

Table 3. Experimental conditions, tracer-determined cation efflux and the index n for gramicidin A-treated lipid bilayers in 10^{-1} M CsCl solutions

Membrane area (cm^2)	Potential applied (mV)	Specific conductivity ($S \cdot cm^{-2}$)	Tracer determined efflux ($mol \cdot cm^{-2} \cdot s^{-1}$)	n
1.0	0	$4.5 \cdot 10^{-6}$	$7.39 \cdot 10^{-13}$	1.6
1.4	0	$5.9 \cdot 10^{-6}$	$9.53 \cdot 10^{-13}$	1.6
1.4	0	$1.1 \cdot 10^{-5}$	$1.71 \cdot 10^{-12}$	1.7
1.4	28	$5.8 \cdot 10^{-6}$	$2.07 \cdot 10^{-12}$	1.5
1.4	24	$6.6 \cdot 10^{-6}$	$1.99 \cdot 10^{-12}$	1.9
	−24		$3.51 \cdot 10^{-13}$	
1.4	−20	$7.4 \cdot 10^{-6}$	$4.80 \cdot 10^{-13}$	1.8
			mean and SD	1.68 ± 0.15

Table 4. Experimental conditions, tracer-determined cation efflux and the index n for gramicidin A-treated lipid bilayers in 10^{-1} M NaCl solutions

Membrane area (cm^2)	Potential applied (mV)	Specific conductivity ($S \cdot cm^{-2}$)	Tracer-determined efflux ($mol \cdot cm^{-2} \cdot s^{-1}$)	n
1.9	0	$1.1 \cdot 10^{-7}$	$2.67 \cdot 10^{-14}$	1.1
1.9	0	$2.1 \cdot 10^{-7}$	$4.69 \cdot 10^{-14}$	1.2
1.7	0	$2.9 \cdot 10^{-7}$	$6.01 \cdot 10^{-14}$	1.3
1.5	0	$4.5 \cdot 10^{-7}$	$1.26 \cdot 10^{-13}$	0.9
1.3	0	$1.7 \cdot 10^{-6}$	$3.56 \cdot 10^{-13}$	1.2
1.3	0	$4.2 \cdot 10^{-6}$	$8.20 \cdot 10^{-13}$	1.4
1.3	0	$7.4 \cdot 10^{-6}$	$1.51 \cdot 10^{-12}$	1.3
1.9	24	$2.5 \cdot 10^{-7}$	$1.03 \cdot 10^{-13}$	1.3
	−24		$3.16 \cdot 10^{-14}$	
1.3	−30	$4.6 \cdot 10^{-7}$	$5.55 \cdot 10^{-14}$	1.1
1.3	−21	$9.9 \cdot 10^{-7}$	$1.70 \cdot 10^{-13}$	1.0
1.3	−32	$1.8 \cdot 10^{-6}$	$7.61 \cdot 10^{-13}$	1.3
1.3	29	$1.9 \cdot 10^{-6}$	$7.16 \cdot 10^{-13}$	1.3
	−29		$1.71 \cdot 10^{-13}$	
1.3	−26	$2.0 \cdot 10^{-6}$	$1.97 \cdot 10^{-13}$	1.3
1.3	−26	$4.3 \cdot 10^{-6}$	$6.53 \cdot 10^{-13}$	1.0
1.3	21	$4.4 \cdot 10^{-6}$	$1.40 \cdot 10^{-12}$	1.4
			mean and SD	1.21 ± 0.15

2. No significant difference was detected between the mean values of the index n determined in the zero voltage condition and at potentials up to 33 mV applied to the membranes. That enabled us to combine data on the index n obtained at zero and nonzero potential differences at the membranes.

3. The main conclusion that may be derived from this set of experiments is the concentration dependence of the index n. The mean values for n presented in Table 2 show that there was an increase of the index from 1.64 ± 0.12 found for the membranes in 2.2×10^{-3} M RbCl to 2.04 ± 0.15 in 10^{-2} M RbCl. The difference is significant at 99% confidence level when estimated with appropriate Student's t-factors. No significant difference was noted between the mean values of n for a series of membranes in 10^{-2} and 10^{-1} M RbCl (2.04 ± 0.15 and 1.99 ± 0.21, respectively). The further increase of the salt concentration to 1.0 M resulted in a decrease of the index n down to 1.47 ± 0.15. This decrease of the n value is statistically reliable at the 99.9% confidence level.

It is known that channels formed by gramicidin A dimers in lipid bilayers possess a low selectivity for alkaline cations (Myers & Haydon, 1972). For 0.1 M chloride solutions the potentiometrically determined permeability ratio was found: $P_{Na}/P_{Rb}/P_{Cs} = 1:5.5:5.8$. Cation selectivity established from single-channel conductance measurements: $G_{Na}/G_{Rb}/G_{Cs} = 1:2.9:2.9$ appeared about two times smaller. One should not expect pronounced changes in the n values with substitution of Rb^+ or Cs^+ ions by Na^+ if low cation selectivity of the channel is predominantly determined by the affinity of cation binding sites of the channel. In that case the loading of the channel at a given concentration of the above cations should not much differ. On the other hand, dependence of the single gramicidin A channel conductance on the concentration of Na^+ ions in solutions seems to be different from that for Cs^+ and Rb^+ ions (Hladky & Haydon, 1972; Bamberg, Noda, Gross & Läuger, 1976; Neher, Sandblom & Eisenman, 1978; Hladky et al., 1979; *cf.* Urry et al., 1980). A simple saturation type of the concentration dependence established for the membranes in sodium salt solutions is in agreement with the concept of gramicidin A channel occupied mostly by one ion, when the dependence with a maximum found for Cs^+ and Rb^+ salt solutions suggested a multiple ion occupancy of the channel. If the supposition concerning the origin of the difference in the mode of conductance-concentration dependence is correct there should also be a pronounced difference in the index n values for the Na^+-loaded channel if compared with that containing Rb^+ or Cs^+.

Data presented in Table 3 showed that the mean values of the index n for membranes in 10^{-1} M CsCl are smaller than those for the membranes in RbCl at the same concentration. The difference is, however, significant only at the 90% confidence level. Value of the index n found for membranes in 10^{-1} M NaCl (*see* Table 4) appears to be much lower than that for the membranes in 10^{-1} M RbCl solution. Even the highest individual value of the index n for membranes in NaCl

solution ($n=1.4$) is 0.2 lower than the lowest n value in the series for membranes in 10^{-1} M RbCl. The mean value of n for membranes in 10^{-1} M NaCl (1.21 ± 0.15) differed from that for membranes in RbCl and CsCl solutions of the same concentration at a confidence level better than 99%. It should be noted that the mean value of the index n found for membranes in NaCl solution is also different from unity at a confidence level better than 99.9%.

To understand the mode of ion translocation through gramicidin A channels it would be desirable to know the position of ion binding sites along the channel. Unfortunately, no certain concept concerning the position of energy wells for cations passing the channel was developed on the basis of the chemical structure of now a well-documented head-to-head π^6(LD) helical dimer model of gramicidin A pore (Urry, 1971, 1972; Urry et al., 1971; Bamberg et al., 1979; Weinstein et al., 1979) as well as for a double helical model of the dimer (Veatch, Mathies, Eisenberg & Stryer, 1974). In this context, O-pyromellitylgramicidin, an analog bearing three charged carboxylic groups at the hydroxyl end of the molecule (Apell, Bamberg, Alpes & Läuger, 1977) shows a certain advantage. When a dimer of the analog spans the bilayer a most probable localization of negatively charged groups and thus possible cation binding sites is at the membrane-solution interface. The properties of O-pyromellitylgramicidin A-modified bilayers prepared from bulk ox brain lipids with the addition of cholesterol were practically the same as those described for bilayers formed of dioleoylphosphatidylcholine (Apell et al., 1977). The results of determinations of the index n for O-pyromellitylgramicidin A-treated membranes in 10^{-1} M RbCl solution are presented in Table 5. The mean value of the index n was nearly the same (1.92 ± 0.10) as that for the membranes treated by uncharged gramicidin A.

The mechanism of cation transport through hydrophilic channels like that formed by gramicidin A dimers in bilayers is commonly compared with another mechanism of ion translocation across lipid bilayer membranes by mobile carriers such as valinomycin and macrotetralides. The theory of mobile carrier ion transport (Markin & Chizmadjev, 1974) predicted that the index n, with the increase of concentration of transported ions in solutions, must deviate from the unity and approach zero. These theoretically predicted changes in the n value are known in biology as an exchange diffusion phenomenon (Ussing, 1949; Kedem & Essig, 1965) used for the explanation of the ratio

Table 5. Experimental conditions, tracer-determined cation efflux and the index n for O-pyromellitylgramicidin A-treated lipid bilayers in 10^{-1} M RbCl solutions

Membrane area (cm^2)	Potential applied (mV)	Specific conductivity ($S\cdot cm^{-2}$)	Tracer-determined efflux ($mol\cdot cm^{-2}\cdot s^{-1}$)	n
1.2	0	$3.3\cdot10^{-6}$	$4.65\cdot10^{-13}$	1.9
1.4	0	$9.3\cdot10^{-6}$	$1.22\cdot10^{-12}$	2.0
1.4	0	$1.3\cdot10^{-5}$	$1.82\cdot10^{-12}$	2.0
1.4	−14	$8.4\cdot10^{-6}$	$7.17\cdot10^{-13}$	1.8
1.4	20	$8.6\cdot10^{-6}$	$2.13\cdot10^{-12}$	1.8
	−20		$5.14\cdot10^{-13}$	
1.4	25	$8.8\cdot10^{-6}$	$2.72\cdot10^{-12}$	2.0
			mean and SD	1.92 ± 0.10

P_i/P_i^* (*see* Eq. 5) smaller than unity. The exchange diffusion phenomenon was not so far tested on artificial bilayer membranes treated by ionophore of a known chemical structure. It was of interest to prove that changes in the n values, with concentration of transported cations in solutions for mobile carrier mechanism, are opposite to those for hydrophilic single-file channels. The results obtained for bilayer membranes modified by valinomycin (concentrations in water solutions were varied from 10^{-8} to 5×10^{-7} M) are presented in Table 6. The values of the index n were found to be close to unity for the membranes in 10^{-2} to 5×10^{-2} M RbCl solutions while for the membranes in 10^{-1} M RbCl it was 0.54. The decrease of the n value with increase of RbCl concentration up to one-tenth molar was significant at the 99% confidence level as compared with the membranes in 10^{-2} to 5×10^{-2} M RbCl. The experiments performed on valinomycin modified membranes in 10^{-1} and 10^{-2} M CsCl solutions showed that the n values at both of these concentrations were close to unity.

Discussion

The results obtained in this work show that unidirectional flux ratio for cations determined for modified bilayer membranes may deviate from unity in opposite directions. For membranes modified by valinomycin the index n was equal to unity (1.00 ± 0.15) for all concentrations of RbCl in solutions smaller than 10^{-1} M. For membranes in the 10^{-1} M solution of the salt the mean value of the index n was 0.54 ± 0.16 which was certainly different from unity. From the data presented by Eyal and Rechnitz (1971) the stability constant for the valinomycin-Rb^+ complex in water should be of

Table 6. Experimental conditions, tracer-determined cation efflux and the index n for lipid bilayers modified by valinomycin

Salt	Salt concentration (M)	Membrane area (cm^2)	Potential applied (mV)	Specific conductivity ($S \cdot cm^{-2}$)	Tracer-determined efflux ($mol \cdot cm^{-2} \cdot s^{-1}$)	n
RbCl	10^{-2}	2.2	0	$5.5 \cdot 10^{-7}$	$1.15 \cdot 10^{-13}$	1.2
	10^{-2}	2.0	0	$9.4 \cdot 10^{-7}$	$2.33 \cdot 10^{-13}$	1.1
	10^{-2}	2.0	0	$1.1 \cdot 10^{-6}$	$3.22 \cdot 10^{-13}$	0.9
	10^{-2}	2.2	0	$1.2 \cdot 10^{-6}$	$2.44 \cdot 10^{-13}$	1.2
	10^{-2}	1.6	−34	$4.3 \cdot 10^{-7}$	$4.82 \cdot 10^{-14}$	1.1
	10^{-2}	1.6	−35	$3.7 \cdot 10^{-7}$	$6.68 \cdot 10^{-14}$	0.8
	10^{-2}	2.0	−37	$9.1 \cdot 10^{-7}$	$7.90 \cdot 10^{-14}$	1.2
	10^{-2}	2.0	−38	$8.3 \cdot 10^{-7}$	$8.39 \cdot 10^{-14}$	1.1
	10^{-2}	2.2	−39	$1.2 \cdot 10^{-6}$	$1.77 \cdot 10^{-13}$	0.9
	10^{-2}	2.0	39 −39	$8.8 \cdot 10^{-7}$	$3.62 \cdot 10^{-13}$ $8.57 \cdot 10^{-14}$	0.9
	10^{-2}	2.2	−56	$7.5 \cdot 10^{-7}$	$9.05 \cdot 10^{-14}$	0.8
					mean and SD	1.02 ± 0.16
	$2 \cdot 10^{-2}$	1.6	0	$7.6 \cdot 10^{-7}$	$2.16 \cdot 10^{-13}$	1.1
	$5 \cdot 10^{-2}$	2.1	0	$1.7 \cdot 10^{-6}$	$4.06 \cdot 10^{-13}$	1.1
	$5 \cdot 10^{-2}$	2.1	−42	$1.9 \cdot 10^{-6}$	$2.86 \cdot 10^{-13}$	0.8
	$5 \cdot 10^{-2}$	2.1	45 −45	$1.7 \cdot 10^{-6}$	$9.34 \cdot 10^{-13}$ $2.34 \cdot 10^{-13}$	0.8
	$5 \cdot 10^{-2}$	2.1	48	$1.4 \cdot 10^{-6}$	$8.59 \cdot 10^{-13}$	0.8
					mean and SD	0.88 ± 0.15
	10^{-1}	1.9	0	$2.4 \cdot 10^{-6}$	$1.14 \cdot 10^{-12}$	0.6
	10^{-1}	1.9	0	$3.4 \cdot 10^{-6}$	$1.51 \cdot 10^{-12}$	0.6
	10^{-1}	2.1	0	$3.7 \cdot 10^{-6}$	$2.21 \cdot 10^{-12}$	0.4
	10^{-1}	1.9	0	$4.8 \cdot 10^{-6}$	$3.21 \cdot 10^{-12}$	0.4
	10^{-1}	2.1	0	$2.3 \cdot 10^{-5}$	$7.34 \cdot 10^{-12}$	0.8
	10^{-1}	2.1	40	$6.0 \cdot 10^{-6}$	$4.16 \cdot 10^{-12}$	0.6
	10^{-1}	2.1	44	$9.5 \cdot 10^{-6}$	$6.29 \cdot 10^{-12}$	0.7
	10^{-1}	1.9	−48	$2.6 \cdot 10^{-6}$	$1.04 \cdot 10^{-12}$	0.4
	10^{-1}	1.9	51 −51	$2.6 \cdot 10^{-6}$	$1.96 \cdot 10^{-12}$ $9.91 \cdot 10^{-13}$	0.3
	10^{-1}	1.9	53	$2.7 \cdot 10^{-6}$	$2.05 \cdot 10^{-12}$	0.6
					mean and SD	0.54 ± 0.16
	$5 \cdot 10^{-1}$	1.4	0	$1.4 \cdot 10^{-6}$	$8.50 \cdot 10^{-13}$	0.4
CsCl	10^{-2}	2.6	0	$1.3 \cdot 10^{-7}$	$3.17 \cdot 10^{-14}$	1.1
	10^{-2}	1.8	0	$6.5 \cdot 10^{-7}$	$1.46 \cdot 10^{-13}$	1.0
	10^{-2}	1.8	−46	$5.5 \cdot 10^{-7}$	$3.62 \cdot 10^{-14}$	1.2
	10^{-2}	1.8	49 −49	$3.6 \cdot 10^{-7}$	$1.84 \cdot 10^{-13}$ $2.20 \cdot 10^{-14}$	1.1
					mean and SD	1.10 ± 0.08
	10^{-1}	2.3	0	$1.6 \cdot 10^{-6}$	$4.65 \cdot 10^{-13}$	0.9
	10^{-1}	2.3	0	$1.8 \cdot 10^{-6}$	$4.30 \cdot 10^{-13}$	1.1
	10^{-1}	2.3	0	$3.1 \cdot 10^{-6}$	$8.20 \cdot 10^{-13}$	1.0
	10^{-1}	2.3	0	$4.4 \cdot 10^{-6}$	$2.26 \cdot 10^{-12}$	0.5
	10^{-1}	2.0	49	$2.8 \cdot 10^{-7}$	$1.56 \cdot 10^{-13}$	1.1
	10^{-1}	2.1	−56	$1.8 \cdot 10^{-6}$	$1.02 \cdot 10^{-13}$	0.8
	10^{-1}	2.7	76	$1.6 \cdot 10^{-7}$	$1.36 \cdot 10^{-13}$	0.8
					mean and SD	0.89 ± 0.21

about $4\ \text{M}^{-1}$. Hence the changes in the index n occur at a RbCl concentration, which is large enough for a significant portion of valinomycin in the water solution to be in a form of complex with the cation. More detailed studies on the dependence of the index n on the concentrations of different complex-forming cations are needed to investigate whether the complex formation in water solutions or at a membrane surface determines changes in the value of the index n. In any case, the phenomenon of exchange diffusion predicted by the theory for mobile carrier mechanism was shown in the model membrane system.

Opposite to the findings for valinomycin, bilayers modified by channel-forming compounds (gramicidin A and O-pyromellitylgramicidi A) demonstrated unidirectional flux ratio exponent to be greater than the unity for all electrolyte systems studied. The difference in the ion-transporting mechanisms (and the mechanism of ionic flux interactions) was thus manifested in the direction of the index n deviation from unity.

From the very first studies on the interaction of bidirectional fluxes in potassium channels of the giant squid axon the mean value of the index n was used to detect a number of cations which occur simultaneously in the channel. Calculations in the pioneering work of Hodgkin and Keynes (1955) permitted the conclusion that the number of cations in the potassium channel regarded as an extra narrow pore with a single-file diffusion of cations should be equal to $n-1$ on the condition that all binding sites of the channel were occupied by cations. The "knock on" mechanisms being the only one possible for the case was not considered as realistic, and the authors gave their final results a more elaborate theoretical treatment of ionic diffusion in a single file with vacancies in the pore. According to that treatment, the index n roughly, but not exactly, must be equal to the average number of ions in the file. It was emphasized by the authors that the index n should not be constant unless the pore is saturated and thus only one vacancy is left. Later on, the importance of the degree of saturation of the sites in the pore was revealed by other investigators (Chizmadjev & Aityan, 1977; Hille & Schwarz, 1978). A detailed theoretical analysis of the problem has been recently given by Kohler and Heckmann (1979, 1980). Calculations based on combination of the absolute reaction rate theory and diagrammatic method (*see* Hill & Chen, 1975; Hill, 1977; Kohler & Vollmerhaus, 1980) enabled us to derive a relation between the value of the index n and the number of sites (m) in the pore for several specific cases. For the pore containing no more than one vacancy it was shown that the minimum and maximum of the m value were $m=n$ and $m=n+1$, respectively, depending on the relation of rate constants for particles leaving the pore, entering the pore and translocating between sites in the pore. The general relation between the n and m values was obtained for high concentrations of transported particles in the solutions. It was demonstrated that for the case of a "very full pore" at most two vacancies should be considered. Particular cases where m varied from $n+1$ to $n+3$ were analyzed with m being equal to or greater than 2, 3 or 4.

Gramicidin A channels containing simultaneously 5 or more cations do not look probable since the electrostatic repulsion between 5 charged particles spaced in a 26 Å-long pore is too strong to make the case realistic (*see* Levitt, 1978*a*). Therefore, one must choose between models with 2, 3 and 4 sites for gramicidin A channels.

In conformity with the results obtained by Kohler and Heckmann (1979, 1980) determinations of the unidirectional flux ratio provide a better information on the functional state of a pore if compared to net fluxes of a single permeating component or current measurements. Even more complete information might be gained when the dependence of the index n on the concentration of permeating particles in the solutions varied in a wide range is obtained. If the theoretical approach of Kohler and Heckmann (1979, 1980) is applied to the material presented in this paper on the index n determined for membranes in RbCl solutions of different concentrations the following conclusions can be drawn:

a. Decrease in the n values with lowering of the concentration of RbCl from 10^{-2} to 2.2×10^{-3} M shows that the case $m=n$, and not $m=n+1$ should be chosen on the condition that gramicidin A channels operate as single vacancy pores.

b. Decrease in the value of n from 2 to 1.5 upon the increase of the RbCl concentration from 10^{-1} to 1.0 M testifies in favor of the case of a two-site pore. Such lowering of the index n as a result of the pore transition to a "very full state" should not occur in the four-site pore, since in the latter $n=2$ is not the maximal value of n that precedes its decrease.

The validity of both conclusions is strictly dependent on the reliability of changes of n with RbCl concentration. Hence possible errors in determinations of n values and the statistical significance of the changes in the parameter should be carefully analyzed. The method employed in this work for determining tracer fluxes enables us to

obtain values for the index n within a series of experiments with a reasonably small scatter. With the decrease of RbCl concentration down to 2.2×10^{-3} M and the increase up to 1.0 M changes in n were found significant when the routine statistical treatment was used to estimate corresponding confidence levels. Nevertheless, a possibility of systematic errors in determination of n for the membranes in solutions of different concentrations should be ruled out. A contribution of the baseground tracer permeability of the lipid matrix of bilayers might be one of the most important factors influencing the index n when membranes show only a moderate increment of ionic flux after the addition of gramicidin A.

Special studies on the tracer permeability across unmodified (bare) bilayer membranes made from the same lipid solution we used in this work had been previously carried out in our laboratory (Grinfeldt, Malev & Schagina, 1980). The experiments showed that ionic fluxes across bare membranes in NaCl, KCl, RbCl and CsCl solutions (determined by measuring ^{22}Na, ^{42}K, ^{86}Rb, ^{137}Cs and ^{36}Cl fluxes) were independent from (1) electrical conductance of the membranes, which corresponded to much smaller ionic permeability, (2) RbCl concentration varied from 2.2×10^{-3} to 1.0 M and (3) transmembrane potential difference up to 150 mV. The tracer permeability coefficients of the bare bilayers for the I*a* group cations ranged for different membranes from 1.6×10^{-10} to 10^{-9} cm s^{-1}. Permeability coefficients for chlorine were found equal to or smaller than 4.7×10^{-11} cm s^{-1}. No significant difference in the tracer permeability coefficients of bare bilayers was detected for the cations of I*a* group.

Corrections for the above electrically silent fluxes may be obtained by subtracting values for ionic fluxes determined for membranes before addition of gramicidin A from those measured after the modifier is added.[1] The values of the index n after such correction are presented in the graph "n_{cor}" of Table 2. As might be seen from the comparison of the n and n_{cor} values the above correction did not notably change either the mode of the dependence of the index n on the RbCl concentration or the statistical significance of its deviation from unity.

The absence of any correlation between the index n values and conductivity of gramicidin A-treated membranes in the experimental series is more evidence for the insignificance of baseground tracer permeability for determination of the index n. For example, the mean value of the index n found for two groups of experiments on the membranes in 10^{-1} M RbCl: one with specific conductances varied from 1.6×10^{-6} to 3.0×10^{-6} S cm^{-2} and another with conductivity variation from 6.2×10^{-6} to 1.6×10^{-5} S cm^{-2} gave the mean values of n, 1.96 ± 0.19 and 2.04 ± 0.24, respectively, showing no significant difference.

It has been mentioned that according to up-to-date data on the helical structure of gramicidin A channels there is no reason to suggest a certain location of two specific binding sites in the pore. A number of dipoles associated with the peptide backbone carbonyl oxygens oriented toward the center of the pore may be responsible for a negative potential distributed inside the channel. In this case the multiple binding sites model of gramicidin A channel looks more probable. P. Läuger (1973) developed an m-sites model of the gramicidin channel which however does not allow the explanation of experimental findings of the index n exceeding the unity, the existence of a maximum in the current versus cation concentration curves and the concentration dependence of a cation permeability ratio (*see* Hladky & Haydon, 1972; Urban, Hladky & Haydon, 1978) since the principle condition of the model postulated was of only one cation present in the pore at a time. In more recent papers (Läuger, 1980; Läuger, Stephan & Frehland, 1980) a single binding site model of the channel with a variable structure (two conformational states barrier model, in particular) had been investigated. In the case where a jump of an ion into a binding site is followed by a comparatively slow transition of the site to its more polarized state the model may explain the channel conductance passing through a maximum upon increase of the ionic concentration in solutions. But even in the case of this more elaborate model the index n should not exceed the unity if the condition of a single occupancy of the channel is preserved.

The 3-barrier 4-site multiple occupied models of gramicidin A channel proposed by G. Eisenman (3B4S′ and 3B4S″) and coworkers (Eisenman et al., 1977, 1978; Eisenman, Hägglund, Sandblom & Enos, 1980) imply the existence of two inner cation binding sites and two lateral sites. The latter are supposed to be located either externally (3B4S″) or internally (3B4S′) to the barrier at the mouth of the channel. The result of the studies on concentration dependence of the index n is con-

[1] The correction might look dubious because of possible changes in the lipid structure around gramicidin A channels. But our determinations of ^{36}Cl fluxes before and after addition of gramicidin A to the system showed no appreciable difference. Thus at least for chlorine permeability changes in the lipid structure caused by the addition of the channel-forming compound are of no significance.

sistent with the 3B4S″ but not the 3B4S′ model. As has already been stated, the result of the studies on concentration dependence of the index n does not agree with a 4-site model unless one pair of the outer sites is represented by very shallow energy wells, and thus the model allows for some kind of 2-site approximation.

Calculations based on electrostatic considerations (Parsegian, 1969; Levitt, 1978*a*, *b*) showed that the most probable location of the cation energy wells should be at the membrane interface and thus close to the channel mouth. In the case of lipid bilayers bearing negative charges this cation energy well location is even more probable. That is why it was of interest to determine the surface charge for our lipid preparation and estimate accumulation of cations at the membrane interface.

The surface charge density for membranes formed of bulk brain lipids was determined in our laboratory by Dr. S.A. Tatulian from the data on electrophoretic mobility of multilayer liposomes measured with the aid of an automatic free electrophoresis instrument "Parmoquant-2" (Carl Zeiss Jena, DDR). The negative charge density for the lipid preparation without tetradecane added when immersed in 10^{-1} M RbCl and NaCl solutions was found to be nearly the same varying in the limit of $(2\pm0.1)\times10^{-2}$ C m^{-2}. It corresponds to one unit charge per about 750 Å^2.[2] The value obtained might be different from that for bilayer membranes made of the same lipids because the effect of organic solvent (tetradecane) was not taken into account. The amount of solvent present in the black bilayer membranes is not certain (*see* Pagano, Ruysschaert & Miller, 1972) but one may suggest that the bilayer structure being in equilibrium with a lipid-forming solution at torus is nearly saturated with organic solvent. The only data we found on the influence of solvent (hexadecane) on the surface potential of lipids were those for dimyristoylphosphatidylcholine monolayers (McIntosh, Simon & MacDonald, 1980). A moderate decrease of the surface potential was registered after the addition of hexadecane to the monolayers. In the same work saturation of the lipid bilayer structures by hexadecane at 0.3 molar fraction of the solvent was shown by a scanning calorimetry method. Taking these data into account we determined the electrophoretic mobility for liposomes made of our lipid mixture with an obvious excess of tetradecane. The surface charge found from these measurements was about 1.4×10^{-2} C m^{-2} or one unit charge per 1120 Å^2. Unlike changes in the surface charge caused by addition of organic solvent to dimyristoylphosphatidylcholine monolayers those for bilayers made of bulk ox brain lipids were found much more prominent, reaching 34%.

If thus found charge density is considered constant and equal 1.4×10^{-2} C m^{-2} for all experimental conditions used in this work the surface concentrations of monovalent cations might be estimated for lipid bilayers in solutions of different RbCl concentrations. Using a conventional Debye-Hükkel approach (*see* Apell, Bamberg & Läuger, 1979) we got surface concentrations of Rb^+ ions equal to 5.7×10^{-2}, 7.1×10^{-2}, 2.0×10^{-1} and 1.26 M for a series of bulk RbCl concentrations: 2.2×10^{-3}, 10^{-2}, 10^{-1} and 1.0 M, respectively. At the outer region of the gramicidin A channel mouth the concentrations of Rb^+ should not differ much from those at the lipid layer surface. When the entrance to the channel is represented by a circular disk free of charges with the radius of 10 Å as suggested by Apell and coworkers (1979) the cation concentration at the center of the channel mouth for the above series of bulk RbCl concentrations should be equal to 3.6×10^{-2}, 4.1×10^{-2}, 1.3×10^{-1} and 1.0 M, respectively. Concentrations thus found differ less from correspondent concentrations of Rb^+ ions in the bulk solutions compared with the cation concentrations at the surface on unperturbed lipid bilayer regions. An effective cation concentration at the entrance of the channel should be somewhere in-between that in the center of the pore mouth and at the lipid surface.

If to accept the above corrections for the surface concentrations the steepness of the decrease in the index n values upon the increase of effective Rb^+ concentration at the pore entrance would be nearly the same as that with the increase of the bulk concentration of the cations. On the other hand stabilization of the surface concentrations caused by the presence of a surface charge of lipid bilayers (pronounced at the two lowest bulk concentrations used) made it difficult to understand an experimental finding of an obvious decrease in the n values with the bulk concentration of Rb^+ going down to 2.2×10^{-3} M.

The estimations of surface concentrations were made upon assumption of the stability of surface charge of the bilayer, regions near the channel mouth inclusive. But from the calorimetric studies on dipalmitoylphosphatidylcholine (Chapmen, Urbina & Keough, 1974; Chapmen, Cornell, Eliaz & Perry, 1977) it is known that incorporation of gramicidin A into bilayers produces pronounced changes in thermotropic behavior of the lipid

[2] Surface charge density determined in the same way for multilayer liposomes made of bulk brain lipids without special addition of cholesterol was found to be about 14% higher than for those with cholesterol added in 1:1 (wt/wt) ratio.

pointing out to its disordering around the channel-forming molecules. If the structure of lipid bilayers made of bulk brain lipid mixture is also altered by the incorporation of gramicidin A molecules it may result in a decrease of the surface charge around the channel thus rendering the above calculations of the surface concentrations improper.

The problem discussed is in some connection with the specific properties of O-pyromellitylgramicidin A channels. Three negative charges located at the entrances of channels formed by this compound should provide an accumulation of counterions in a way similar to that caused by the negative surface charge of lipid surroundings. In the energy profile for cations passing the channel three negative fixed charges may be represented by energy wells much deeper than those of uncharged gramicidin channels. Located near the interface these wells are probably in equilibrium with bulk electrolyte solutions. We considered three negative charges of O-pyromellitylgramicidin molecule as randomly distributed at the area of the cross-section of the channel mouth of about 300 $Å^2$. The local charge density in that case must be 1.5×10^{-1} C m^{-2}, which corresponds to the surface concentration of monovalent cations at the channel mouth for membranes in solutions with the 10^{-1} molar bulk concentration of the cations as big as 7 M. The latter should produce a many-fold increase in the rate of cation entrance to the pore when compared with the rate for uncharged gramicidin A channels. Hence the ratio of rates for cations entering the pore and for their translocations inside the pore should also increase many times if there is no change of the translocation process. According to the theory developed by Kohler and Heckmann (1979, 1980) a significant increase in the above ratio results in a decrease in the index n, the fact experimentally proved by the decrease of the index n determined for gramicidin A-treated membranes in 1.0 M RbCl. In spite of a very high local concentration of cations calculated for the entrance of the pore bearing negative charges we did not find experimentally any significant decrease in the index n compared with that for uncharged gramicidin. This disagreement with the theoretical prediction has still not been explained.

Studies on the single channel conductance of O-pyromellitylgramicidin A-modified membranes (Apell et al., 1977) showed that some additional steric restrictions should be considered at the mouth of the channels formed by the compound when correlated with those of uncharged gramicidin A. The authors suggested that this steric restriction is caused by pyromellityl residue obstructing the channel entrance. In the energy profile for cations passing the channel these steric restrictions should be represented by barriers located either before or just behind the above-discussed deep energy wells formed by the presence of negative charges. In both cases the increase in the rate of cations entering the pore due to counter-ion accumulation may be compensated by the appearance of additional barriers. Thus the ratio of rates for cations entering the pore and translocating inside it may not be changed much in comparison with that for uncharged gramicidin channels. Thus no significant decrease in the index n would be observed.

If energy wells for cations representing negative charges at O-pyromellitylgramicidin A channels are located behind the steric restriction barriers the positions of these wells may either coincide with those of the wells formed by pentadecapeptide part of the π^6(LD) helix (common for charged and uncharged gramicidins) or may be different. Depending on that O-pyromellitylgramicidin channels may have a two- or a four-cation binding site structure. As in the case of gramicidin A determinations of the dependence of the index n on the concentrations of permeating cations in solutions may provide information required for a correct choice of the model.

One of the most interesting problems raised by investigations of unidirectional flux ratios is the nature of pronounced difference in the index n values for various cation species. The value of the index n for gramicidin A-modified membranes in 10^{-1} M RbCl solution was nearly two times greater than that for membranes in NaCl of the same concentration. Such cation specificity of the index n may depend either on the conformational change of the channel containing different cations or on the difference in the position of binding sites for cations, which affect the degree of channel loading through electrostatic repulsion. The third possibility is a specific influence of cations on the structure of boundary lipids and thus on the properties of ion-conducting channels. This problem studied by determinations of bidirectional flux interactions for gramicidin A channels for two different cations simultaneously at their various molar ratios in solutions will be described in our following paper.

The authors are greatly indebted to Drs. K. Heckmann and H. Kohler for helpful discussion and Drs. S.A. Tatulian and V.V. Malev for their help in experimental work.

References

Andersen, O.S., Procopio, J. 1978. Ion entry into gramicidin A channels. *Biophys. J.* **17**:26a

Andersen, O.S., Procopio, J. 1980. Ion movement through gramicidin A channels. *Acta Physiol. Scand.* (Suppl.) **481**:27–35

Apell, H.J., Bamberg, E., Alpes, H.L., Läuger, P. 1977. Formation of ion channels by a negatively charged analog of gramicidin A. *J. Membrane Biol.* **31**:171–188

Apell, H.J., Bamberg, E., Läuger, P. 1979. Effect of surface charge on the conductance of the gramicidin channel. *Biochim. Biophys. Acta* **552**:369–379

Bamberg, E., Alpes, H.L., Apell, H.J., Bradley, R., Härter, B., Auelle, M.J., Urry, D.W. 1979. Formation of ionic channels in black lipid membranes by succinic derivates of gramicidin A. *J. Membrane Biol.* **50**:257–270

Bamberg, E., Apell, H.J., Alpes, H., Gross, E., Morell, J.L., Harbaugh, J.F., Janko, K., Läuger, P. 1978. Ion channels formed by chemical analogs of gramicidin A. *Fed. Proc.* **37**:2633–2638

Bamberg, E., Janko, K. 1977. The action of carbon-suboxide dimerized gramicidin A on lipid bilayer membranes. *Biochim. Biophys. Acta* **465**:486–498

Bamberg, E., Noda, K., Gross, E., Läuger, P. 1976. Single-channel parameters of gramicidin A, B, and C. *Biochim. Biophys. Acta* **419**:223–228

Begenisich, T., DeWeer, P. 1977. Ionic interactions in the potassium channel of squid giant axons. *Nature (London)* **269**:710–711

Begenisich, T., DeWeer, P. 1980. Potassium flux ratio in voltage-clamped squid giant axons. *J. Gen. Physiol.* **76**:83–98

Behn, V. 1897. Über wechselseitige Diffusion von Electrolyten in verdünnten wässeringen Losungen insbesondere über Diffusion gegen das Concentrationsgefälle. *Ann. Phys. Chem. (N.F.)* **62**:54–67

Cahalan, M., Begenisich, T. 1976. Sodium channel selectivity. Dependence on internal permeant ion concentration. *J. Gen. Physiol.* **68**:111–125

Chandler, W.K., Hodgkin, A.L., Meves, H. 1965. The effect of changing the internal solution on sodium inactivation and related phenomena in giant axons. *J. Physiol. (London)* **180**:821–836

Chandler, W.K., Meves, H. 1965. Voltage clamp experiments on internally perfused giant axons. *J. Physiol. (London)* **180**:788–820

Chapmen, D., Cornell, B.A., Eliaz, A.W., Perry, A. 1977. Interactions of helical polypeptide segments which span the hydrocarbon region of lipid bilayers. Studies of the gramicidin A lipid-water system. *J. Mol. Biol.* **113**:517–538

Chapmen, D., Urbina, J., Keough, K.M. 1974. Biomembrane phase transitions. Studies of lipid-water systems using differential scanning calorimetry. *J. Biol. Chem.* **249**:2512–2521

Chizmadjev, Y.A., Aityan, S.K. 1977. Ion transport across sodium channels in biological membranes. *J. Theor. Biol.* **64**:429–453

Eisenman, G., Hägglund, J., Sandblom, J., Enos, B. 1980. The current-voltage behavior of ion channels: Important features of the energy profile of the gramicidin channel deduced from the conductance-voltage characteristic in the limit of low ion concentration. *Uppsala J. Med. Sci.* **85**:247–257

Eisenman, G., Sandblom, J., Neher, E. 1977. Ionic selectivity, saturation, binding, and block in the gramicidin A channel: A preliminary report. *In:* Metal-Ligand Interactions in Organic Chemistry and Biochemistry. Part 2. B. Pullman N. Goldblum, editors. pp. 1–36. D. Reidel Publishing Company, Dordecht, Holland

Eisenman, G., Sandblom, J., Neher, E. 1978. Interactions in cation permeation through the gramicidin channel: Cs, Rb, K, Na, Li, Tl, H, and effects of anion binding. *Biophys. J.* **22**:307–340

Eyal, E., Rechnitz, G.A. 1971. Mechanistic studies on the valinomycin-based potassium electrode. *Anal. Chem.* **43**:1090–1093

Grinfeldt, A.E., Malev, V.V., Schagina, L.V. 1980. Tracer permeability of lipid bilayer membranes. *Cytology* **22**:182–188 (*In Russian*)

Gross, E., Witkop, B. 1965. Gramicidin. IX. Preparation of gramicidin A, B, and C. *Biochemistry* **4**:2495–2501

Hagiwara, S., Miyazaki, S., Rosenthal, N.P. 1976. Potassium current and the effect of cesium on this current during anomalous rectification of the egg cell membrane of a starfish. *J. Gen. Physiol.* **67**:621–638

Hagiwara, S., Yoshii, M. 1979. Effects of internal potassium and sodium on the anomalous rectification of the starfish egg as examined by internal perfusion. *J. Physiol. (London)* **292**:251–256

Heckmann, K. 1965*a*. Zur Theorie der "Single File"-Diffusion. I. *Z. Phys. Chem. (N.F.)* **44**:184–203

Heckmann, K. 1965*b*. Zur Theorie der "Single File"-Diffusion. II. *Z. Phys. Chem. (N.F.)* **46**:1–25

Heckmann, K. 1968. Zur Theorie der "Single File"-Diffusion. III. Sigmoide Konzentrationsabhängigkeit unidirectionaler Flüsse bei "Single File" Diffusion. *Z. Phys. Chem. (N.F.)* **58**:206–219

Heckmann, K. 1972. Single file diffusion. *In:* Biomembranes. K. Kreuzer & J.F.G. Slegers, editors. Vol. 3, pp. 127–153. Plenum Publishing Corp., New York

Heckmann, K., Volmerhaus, W. 1970. Zur Theorie der "Single File"-Diffusion. IV. Vergleich von Leerstellendiffusion und "knock on"-Mechanismus. *Z. Phys. Chem. (N.F.)* **71**:320–328

Heckmann, K., Vollmerhaus, W., Kutschera, J., Vollmerhaus, E. 1969. Mathematische Modelle für reaktionskinetische Phänomene. *Z. Naturforsch., Teil A.* **24**:664–673

Hill, T.L. 1977. Free Energy Transduction in Biology. pp. 1–201. Academic Press, Inc., New York, San Francisco, London

Hill, T.L., Chen, Y. 1975. Stochastics of cycle completion (fluxes) in biochemical kinetic diagrams. *Proc. Natl. Acad. Sci. USA* **72**:1291–1295

Hille, B. 1975*a*. Ionic selectivity of Na and K channels of nerve membranes. *In:* Membranes: A Series of Advances. G. Eisenman, editor. Vol. 3, pp. 255–323. Marcel Dekker, Inc., New York

Hille, B. 1975*b*. Ionic selectivity, saturation and block in sodium channels. A four barrier model. *J. Gen. Physiol.* **66**:535–560

Hille, B., Schwarz, W. 1978. Potassium channels as multi-ion single-file pores. *J. Gen. Physiol.* **72**:409–442

Hladky, S.B., Haydon, D.A. 1972. Ion transfer across lipid membranes in the presence of gramicidin A. I. Studies of the unit conductance channel. *Biochim. Biophys. Acta* **274**:294–312

Hladky, S.B., Urban, B.W., Haydon, D.A. 1979. Ion movements in pores formed by gramicidin A. *In:* Membrane Transport Processes. C.F. Stevens and R.W. Tsien, editors. Vol. 3, pp. 89–103. Raven Press, New York

Hodgkin, A.L., Huxley, A.F. 1952. Currents carried by sodium and potassium ions through the membrane of the giant axon of *Loligo*. *J. Physiol. (London)* **116**:449–472

Hodgkin, A.L., Keynes, R.D. 1955. The potassium permeability of a giant nerve fibre. *J. Physiol. (London)* **128**:61–88

Horowicz, P., Gage, P.W., Eisenberg, R.S. 1968. The role of the electrochemical gradient in determining potassium fluxes in frog striated muscle. *J. Gen. Physiol.* **51**:193s–203s

Jung, C.G. 1971. Permeability of bimolecular membranes made from lipid extracts of human red cell ghosts to sugars. *J. Membrane Biol.* **5**:200–214

Kedem, O., Essig, A. 1965. Isotope flows and flux ratios in biological membranes. *J. Gen. Physiol.* **48**:1047–1070

Kohler, H.-H., Heckmann, K. 1979. Unidirectional fluxes in saturated single-file pores of biological and artificial membranes. I. Pores containing no more than one vacancy. *J. Theor. Biol.* **79**:381–401

Kohler, H.-H., Heckmann, K. 1980. Unidirectional fluxes in saturated single-file pores of biological and artificial membranes. II. Asymptotic behavior at high degree of saturation. *J. Theor. Biol.* **85**:575–595

Kohler, H.-H., Vollmerhaus, E. 1980. The frequency of cyclic processes in biological multistate systems. *J. Math. Biol.* **9**:275–290

Kostyuk, P.G., Krishtal, O.A. 1977. Effects of calcium and calcium-chelating agents on the inward and outward current in the membrane of mollusc neurones. *J. Physiol. (London)* **270**:569–580

Kübel, M. 1975. Electrical conductivity of some hydrophilic ions in spherical bimolecular lipid membranes. *Pro. Colloid Polymer Sci.* **57**:133–140

Läuger, P. 1973. Ion transport through pores: A rate theory analysis. *Biochim. Biophys. Acta* **311**:423–441

Läuger, P. 1980. Kinetic properties of ion carriers and channels. *J. Membrane Biol.* **57**:163–178

Läuger, P., Stephan, W., Frehland, E. 1980. Fluctuations of barrier structure in ionic channels. *Biochim. Biophys. Acta* **602**:167–180

Levitt, D.G. 1978*a*. Electrostatic calculations for an ion channel. I. Energy and potential profiles and interactions between ions. *Biophys. J.* **22**:209–219

Levitt, D.G. 1978*b*. Electrostatic calculations for an ion channel. II. Kinetic behavior of the gramicidin A channel. *Biophys. J.* **22**:221–248

Markin, V.S., Chizmadjev, Y.A. 1974. Induced Ionic Transport. Nauka, Moscow. 251 pp. (*In Russian*)

McIntosh, T.J., Simon, S.A., MacDonald, R.C. 1980. The organization of *n*-alkanes in lipid bilayers. *Biochim. Biophys. Acta* **597**:445–463

Meves, H., Vogel, W. 1973. Calcium inward currents in internally perfused giant axon. *J. Physiol. (London)* **235**:225–265

Myers, V.B., Haydon, D.A. 1972. Ion transfer across lipid membranes in the presence of gramicidin A. II. The ion selectivity. *Biochim. Biophys. Acta* **274**:313–322

Neher, E., Sandblom, J., Eisenman, G. 1978. Ionic selectivity, saturation, and block in gramicidin A channels. II. Saturation behavior of single channel conductances and evidence for the existence of multiple binding sites in the channel. *J. Membrane Biol.* **40**:97–116

Pagano, R.E., Ruysschaert, J.M., Miller, I.R. 1972. The molecular composition of some lipid bilayer membranes in aqueous solution. *J. Membrane Biol.* **10**:11–30

Parsegian, V.A. 1969. Energy of an ion crossing a low dielectric membrane: Solutions to four relevant electrostatic problems. *Nature (London)* **221**:844–846

Rosenberg, P.A., Finkelstein, A. 1978. Interaction of ions and water in gramicidin A channels. *J. Gen. Physiol.* **72**:327–340

Sandblom, J., Eisenman, G., Neher, E. 1977. Ionic selectivity, saturation, and block in gramicidin A channels. I. Theory for the electrical properties of ion selective channels having two pairs of binding sites and multiple conductance states. *J. Membrane Biol.* **31**:383–417

Schagina, L.V., Grinfeldt, A.E., Lev, A.A. 1978. Interaction of cation fluxes in gramicidin A channels in lipid bilayer membranes. *Nature (London)* **273**:243–245

Schagina, L.V., Grinfeldt, A.E., Lev, A.A. 1980. Study of the interaction between cation fluxes in bilayer phospholipid membranes modified with gramicidin A. Determination of the number of ion-binding sites and their location in gramicidin channel. *Biophysica* **25**:648–653 *(In Russian)*

Teorell, T. 1935. An attempt to formulate a quantitative theory of membrane permeability. *Proc. Soc. Exp. Biol. Med.* **33**:282–285

Urban, B.W., Hladky, S.B., Haydon, D.A. 1978. The kinetics of ion movements in the gramicidin channel. *Fed. Proc.* **37**:2628–2632

Urban, B.W., Hladky, S.B., Haydon, D.A. 1980. Ion movement in gramicidin pores. An example of single-file transport. *Biochim. Biophys. Acta* **602**:331–354

Urry, D.W. 1971. The gramicidin A transmembrane channel: A proposed π(LD) helix. *Proc. Natl. Acad. Sci. USA* **68**:672–676

Urry, D.W. 1972. Conformation of protein in biological membranes and a model transmembrane channel. *Ann. N.Y. Acad. Sci.* **159**:108–125

Urry, D.W., Goodall, M.C., Glickson, J.D., Mayers, D.C. 1971. The gramicidin A transmembrane channel: Characteristics of head to head dimerized π(L, D) helices. *Proc. Natl. Acad. Sci. USA* **68**:1907–1911

Urry, D.W., Venkatachalam, C.M., Spisni, A., Bradley, R.J., Trapane, T.L., Prasad, K.U. 1980. The malonyl gramicidin channel: NMR-derived rate constants and comparison of calculated and experimental single-channel currents. *J. Membrane Biol.* **55**:29–51

Ussing, H.H. 1949. The distinction by means of tracers between active transport and diffusion. *Acta Physiol. Scand.* **19**:43–56

Veatch, W., Mathies, R., Eisenberg, M., Stryer, L. 1974. Simultaneous fluorescence and conductance studies of planar bilayer membranes containing a highly active and fluorescent analog of gramicidin A. *J. Mol. Biol.* **99**:75–92

Weinstein, S., Wallace, B., Blout, E.R., Morrow, J.S., Veatch, W. 1979. Conformation of gramicidin A channel in phospholipid vesicles: a ^{13}C and ^{19}F nuclear magnetic resonance study. *Proc. Natl. Acad. Sci. USA* **76**:4230–4234

Received 11 June 1982; revised 5 October 1982