J. Membrane Biol. 68, 79–88 (1982)

The Journal of
Membrane Biology

Effect of Anions on the Cation Selectivity of Gramicidin-Containing Liposomes

B. Eleazar Cohen

Departamento de Biología Celular, Facultad de Ciencias, Universidad Central de Venezuela, Caracas, Venezuela

Summary. An osmotic method was used to study the salt permeability induced by gramicidin A in liposomes. Sequences of cation permeation were obtained for iodide, salycilate, acetate und formate salts in liposomes below and above their transition temperature. Salycilate and formate salts, unlike acetate and iodide salts, exhibit the same sequences for cation selectivity in liposomes below and above their transition temperature. These results can be explained by assuming three mechanisms for salt permeation across gramicidin-containing liposomes: (i) the anion moves by the lipid part of the membrane whereas the cation moves by the gramicidin channel, (ii) movement of the undissociated acid species occurs through the lipid part of the membrane followed by cation-proton exchange via the gramicidin channel and (iii) the cation and anion may move simultaneously via the gramicidin channel.

When the movement of the anion or undissociated acid across the lipid part of the membrane is not rate limiting the permeation process, the cation selectivity obtained agrees with the cation selectivity of the gramicidin A channel, as determined by others using independent measurements.

Key words gramicidin A · permeability · anions · liposomes · salycilate

Introduction

The use of osmotic methods to study the permeability properties of different solutes diffusing across biological membranes have been limited by the uncertain nature of the interactions between solutes and water molecules within such a structure.

The liposome membrane system (*see* Bangham, Hill & Miller, 1974) is generally considered to be homogeneous with respect to the transport of water and small nonelectrolytes (Cohen & Bangham, 1972). However, in these membranes, it is possible to investigate the characteristics of the simultaneous movement of ions and water molecules by adding a polypeptide such as gramicidin A. This alternative route is a channel formed by two gramicidin molecules, possibly linked by hydrogen bonds in the middle of the membrane (Urry, 1971).

Gramicidin A is a member of a class of antibiotics which exerts its action by increasing the cation permeability of biological membranes (Pressman, 1965; Chappel & Crofts, 1965). This increased cation permeability induced by gramicidin has also been obtained in liposomes (Henderson, McGivan & Chappell, 1969) and black lipid membranes (Mueller & Rudin, 1967; Tosteson, 1968; Goodall, 1971; Hladky & Haydon, 1972).

Relevant information about the mechanism of action of gramicidin A was obtained when Hladky and Haydon (1970) observed that at very low concentration this antibiotic produces well defined step-like fluctuations in the conductance of black lipid membranes. These fluctuations are thought to represent the opening and closing of single conducting channels (Haydon & Hladky, 1972; Lauger, 1973). Additional evidence that gramicidin A acts as a transmembrane channel was provided by experiments in which the "freezing" of a thin lipid film did not appear to affect the conductance induced by the antibiotic (Krasne, Eisenman & Szabo, 1971). Also, an increased transport of water and solutes such as formamide is observed in liposomes below their transition temperature upon incorporation of gramicidin A (Cohen, 1975*a*).

Urry (Urry, 1971; Urry, Goodall, Glickson & Mayers, 1971) has proposed a family of lipophilic, left-handed helical structures, called the $\Pi_{L,D}$ helical structures, as possible models for the gramicidin A channel. An alternative model, in which both peptide chains are coiled around a common axis (Veatch, Fossel & Blout, 1974), is not supported by experimental evidence (Bamberg, Apell & Alpes, 1977).

A number of theoretical treatments have been proposed to describe the transport of ions through these channels (Hladky, 1972; Lauger, 1973; Sandblom, Eisenman & Neher, 1977). Sandblom et al. have proposed a model for the gramicidin channel which extends previous two-site models by adding a specific cationic binding site at each entrance to the channel.

0022-2631/82/0068-0079 $02.00

This four-site model predicts that the membrane potential at zero current should be described by a Goldman-Hodgkin-Katz equation with concentration-dependent permeability ratios (Sandblom et al., 1977).

Eisenman et al. (Eisenman, Sandblom & Neher, 1978) have argued that a multi-site channel model of the type proposed by them allows for the passage of anions, because even though the channel sites are only cation binding, the resulting electrostatic barrier for anions can be reduced by the presence of cations in the channel. In effect, Eisenman et al. original observation was of a 10% permeability of acetate relative to Tl^+ when both outer sites of the channel were loaded significantly with Tl^+ (Eisenman, Sandblom & Neher, 1977). This result has recently been confirmed by Urban, Hladky and Haydon (1980) who show acetate permeabilities ranging from 2–3% of the Tl^+ for somewhat lower (2 mM) Tl^+ concentrations on one side of the channel.

One of the dimeric helical structures proposed by Urry, the $\Pi^6_{L,D}$ helix, seems to be consistent with the cation selectivity of the channel (Haydon & Hladky, 1972) and with the water permeability (Cohen, 1975*a*; Rosenberg & Finkelstein, 1978). The information obtained from the nonelectrolyte selectivity of the channel, as determined in liposomes below their transition temperature (Cohen, 1975*a*), also opens the possibility that small organic ions such as formate and formamidine could penetrate across such a structure.

In the present work, an osmotic method has been used to study the permeability to different salts of liposomes loaded with gramicidin A. The cation selectivity obtained for iodide, salicylate, acetate and formate salts varies with the lipid composition of the liposome system and the anion accompanying the movement of the cation. This suggests that several mechanisms for salt permeation are involved in determining such cation selectivities, including the simultaneous movement of cations and anions through the gramicidin channel.

Materials and Methods

Egg-lecithin was extracted from egg yolks by alumina and silicic acid chromatography according to the procedure of Papahadjopoulos and Miller (1967). Cholesterol, 1,2-dipalmitoyl-L-3-lecithin and dicetylphosphoric acid were obtained from Sigma Chemical Co. Gramicidin A was obtained from Koch-Light. Salts were obtained from British Drug House or prepared from the corresponding acids and bases. The osmolality of the solutions was always checked.

The sodium salts of C^{14}-acetic acid, C^{14}-formic acid, H^3-acetic acid and H^3-formic acid were obtained from the Radiochemical Centre, Amersham, England.

Liposomes were formed in a 20-mM KCl solution, as described previously (Cohen, 1975*a*). Gramicidin A dissolved in methanol was always added to the lipids before the liposomes were formed.

Permeability coefficients to different salts were measured by the osmotic method developed by Hill and Cohen (1972). A full description of this method is given in a previous paper (Cohen, 1975*a*). Briefly, when liposomes are mixed with a hypertonic salt solution, the vesicles shrink as water flows out of the vesicles down its concentration gradient (Fig. 1). The volume goes through a minimum and, as solute followed by water flows into the vesicles, the vesicles increase in volume. Hill et al. have shown that the maximum slope of the volume change after the minimum volume is a good measure of the permeability coefficient of a solute, provided that solute permeates across the membrane much more slowly than water.

In the present work, a stopped-flow instrument (Durrum Instrument, Palo Alto, Calif.) was used to rapidly mix liposomes with a 100-mM solution of the salt investigated. The final (after mixing) concentration of lipid was 1 mM. In order to follow the volume changes, the time course of the transmittance changes (*see* Fig. 1) at 450 nm was monitored on a Tektronix storage oscilloscope equipped with a Polaroid camera. The results reported represent the analysis of at least five measurements of every solute under study.

In order to assess by an independent method, the effect of gramicidin A on the solute permeability of liposomes, the rate of leakage of C^{14} and H^3 labeled acetic and formic acid was measured using the method described by Bangham et al. (1974). For this purpose, two liposome suspensions were prepared from a mixture of 96% dipalmitoyl-lecithin and 4% dicethylphosphoric acid in a 20-mM KCl solution containing 145 μCi/ml of H^3-formic acid (or H^3-acetic acid) and 5 μCi/ml of C^{14}-acetic acid (or C^{14}-formic acid). Gramicidin was added to one of the lipid containing flasks, before adding the aqueous phases to make a final concentration of 50 μg/ml. The preparations were left overnight to equilibrate at room temperature. Each of them was then passed through a G-50 Sephadex column equilibrated with a 20-mM KCl solution. After collecting 8 ml of the turbid effluent, 3-ml aliquots were transferred to two dialysis bags of visking tubing. Finally, visking bags were put into flasks containing 30 ml of the isotope-free 20-mM KCl solution. Samples of the medium (0.5 ml) were removed at 5-min intervals and put into vials containing scintillation fluid (Bray, 1960). Both isotopes were assayed simultaneously – in a Packard scintillation spectrometer.

Results

In order to determine the gramicidin A concentration most suitable for carrying out volume change measurements, the changes in transmittance after the minimum volume of liposomes loaded with gramicidin and suspended in 0.1 M K-acetate solution were recorded (Table 1). The small range of concentrations of gramicidin A used, is due to the fact that it was found that smaller amounts of this ionophore do not produce volume changes large enough to be detected and, at high concentrations, the presence of gramicidin induced a reduction in the optical density of the liposome suspension. For this reason, a gramicidin concentration of 5 μg/μmol of lipid was the value used in all permeability determinations. At this concentration, the dipalmitoyl-lecithin liposomes containing 48% cholesterol (*see* Table 1) exhibit no measurable changes in transmittance after the minimum volume for most of the salts used, with the exception of the potassium iodide solution.

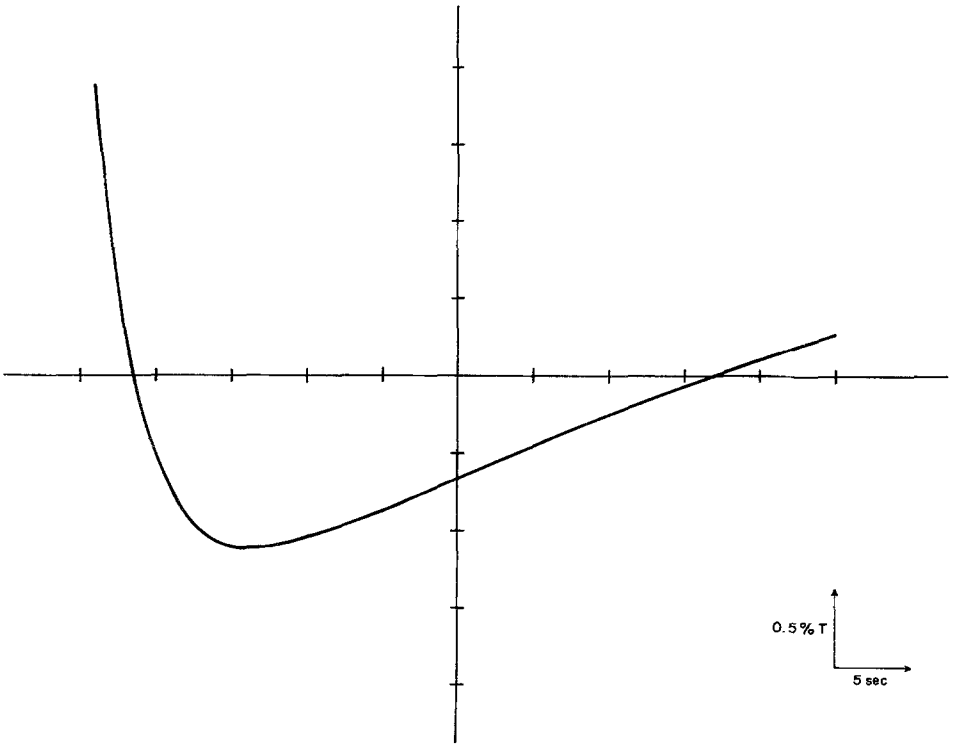


Fig. 1. Transmittance (T) changes of a liposome suspension mixed with an hypertonic potassium iodide solution

Table 1. The effect of different concentrations of gramicidin A on salt permeability across liposome membranes[a]

Salt	Gramicidin A concentration (μg/μmol lipid)	DPL P_{salt}/P_{urea}	DPL + 48% Chol.[b] P_{salt}/P_{urea}
K−Ac	2.5	0.45±0.1	n.m.
K−Ac	5.0	0.78±0.3	n.m.
K−Ac	10.0	1.10±0.3	n.m.
K−I	5.0	*see* Table 2	0.65±0.05
Na−I	5.0	*see* Table 2	n.m.

[a] Temp. = 30 °C; Ac = acetate; n.m. = not measurable by the present method.

[b] This behavior of DPL+48% Chol. liposomes is possibly due to a lower activity of the gramicidin channel in these membranes, as indicated by the observation that the selectivity of the pair formamide/acetamide is only twofold compared with about 14-fold in DPL liposomes at the same gramicidin concentration (100 μg/20 μmol of lipid) (Cohen, 1975*b*).

The Effect of the Length of the Hydrocarbon Chains on the Equilibrium Optical Densities of Gramidicin-Containing Liposomes

Liposomes prepared from saturated lecithins and gramicidin A are structures osmotically active below as well as above the transition temperature of the phospholipids (Cohen, 1975*a*). However, the total optical density changes (at equilibrium) of liposomes loaded with a given amount of gramicidin and kept below their transition temperature (Fig. 2) depend on the length of the hydrocarbon chains of the phospholipids. In Fig. 2, the reciprocal of the optical density changes (proportional to the liposome volume changes) with a negative sign correspond to the liposomes that are suspended in hypertonic solutions of a solute such as erythritol that is impermeable in this system. On the contrary, when liposomes are suspended in a hypertonic solution of a permeable solute such as formamide or in pure water, the corresponding reciprocal of the optical density changes $(O.D.)^{-1}$ are positive, indicating an increase in volume. It can be seen in Fig. 2 that the larger volume changes, whether due to swelling or shrinking of liposomes, are obtained for liposomes prepared with dimyristoyl-lecithin.

Relative Permeabilities to Different Salts in Liposomes Containing Gramicidin A

In the light of these results, the liposomes used to measure the permeabilities to different salts were prepared with the following lipids:

a) dipalmitoyl-lecithin + dicetylphosphoric acid (96:4).

b) egg-phosphatidylcholine + dicetylphosphoric acid (96:4).

c) egg-phosphatidylcholine + cholesterol + dicetylphosphoric acid (48:48:4).

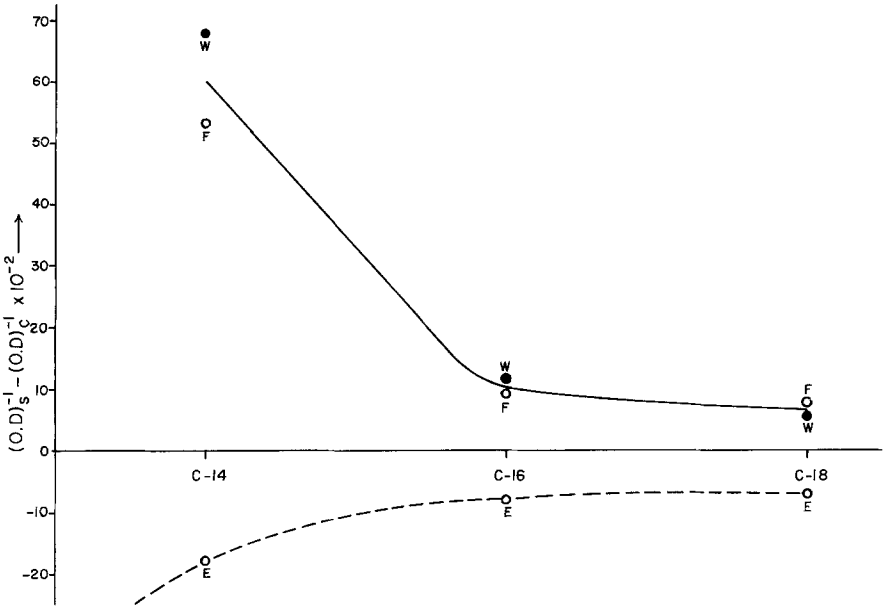


Fig. 2. The effect of the length of the hydrocarbon chains on the equilibrium optical densities of liposomes. *C-14*: dimyristoyl-lecithin; *C-16*: dipalmitoyl-lecithin; *C-18*: distearoyl-lecithin. Liposomes are always formed with a 4 mol % DCP acid and 5 μg gramicidin A/μmol lipid. *Ordinate:* The difference between the inverse of the equilibrium optical density of the solution and the control value (KCl 20 mM). *Abscissa:* the length of the hydrocarbon chains. *F*, formamide; *E*, erythritol; *W*, water

Table 2. Permeability to different salts of gramicidin-containing liposomes[a]

Salt	DPL P_{salt}/P_{urea}	PC P_{salt}/P_{urea}	PC+48% Chol. P_{salt}/P_{urea}
K−Ac	0.78±0.3	0.34±0.10	0.16±0.05
Na−Ac	1.28±0.3	0.28±0.05	0.11±0.01
K−For	2.90±0.5	0.10±0.02	0.10±0.02
Na−For	6.40±0.5	0.18±0.02	0.14±0.01
K−Sal	3.80±0.5	0.65±0.10	0.18±0.02
Na−Sal	7.20±2.0	1.20±0.20	0.22±0.02
KI	0.16±0.03	0.16±0.03	2.00±0.30
NaI	0.14±0.03	0.18±0.02	0.64±0.05
NaI[b]	–	–	1.56±0.10
Form-Ac	3.60±0.1		
Acet-Ac	n.m.		

[a] Temp. = 30 °C. Gramicidin concentration = 5 μg/μmol lipid.
[b] Solution saturated with molecular I_2.
Ac: acetate, For: formate, Sal: salicylate, Form: formamidine, Acet: acetamidine.

The numbers in parentheses were the molar ratios used. Such membrane systems are termed DPL, PC and PC+cholesterol liposomes, respectively. They have been chosen because they do not differ very much in the thickness of the hydrocarbon part of the membranes, and so comparisons between the permeabilities induced by gramicidin A can be done at the same temperature (30 °C).

In Table 2, the permeability values obtained for sodium and potassium acetates, formates, salicylates and iodides are shown. All the values quoted are relative to the permeability of urea, a solute which is permeable in all the systems investigated.

As it can be observed from the values of Table 2, the relative permeabilities for the different salts depend on the lipid composition of the liposome membrane system. Thus, in DPL liposomes the sequence of permeabilities obtained for potassium or sodium salts are as follows (Fig. 3*a*)

salicylates > formates > acetates > iodides.

In PC liposomes (Fig. 3*b*),

salicylates > acetates > iodides > formates

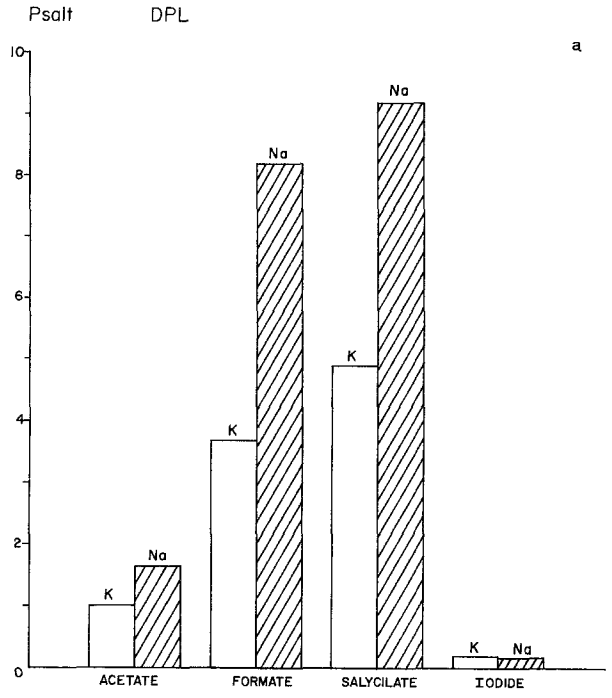
and in PC+cholesterol liposomes, the sequence obtained for K-salts is (Fig. 3*c*).

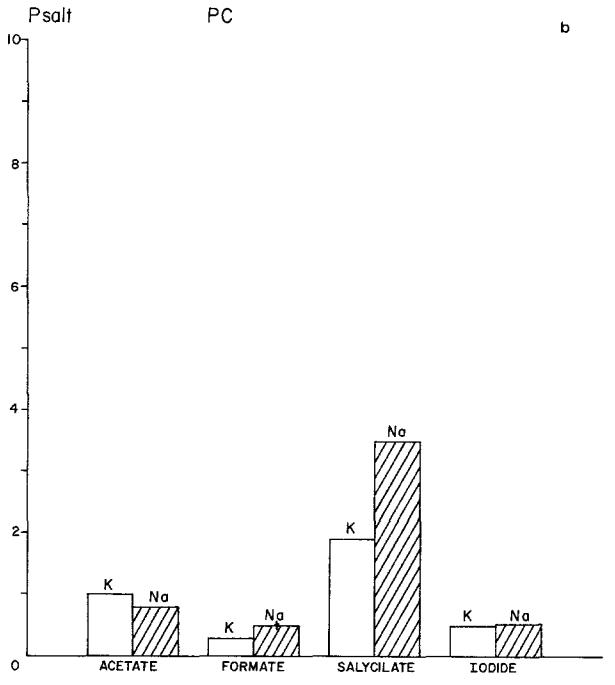
iodide > > salicylate > acetate > formate

and for Na-salts is

iodide > > salicylate > formate > acetate.

It can be seen in Fig. 4 that there are some variations of the selectivity for sodium and potassium with the composition of the lipid system used to prepare the liposomes (Table 2). Thus, sodium salicylate and sodium formate are more permeable than the corresponding potassium salts in all types of liposomes investigated. However, sodium acetate is more permeable than potassium acetate in DPL but less permeable in PC and PC+cholesterol. On the other hand, when iodide salts are used, no significant discrimination between sodium and potassium salts is observed in DPL and PC liposomes (Table 2) but in PC+cholesterol liposomes, potassium iodide is more permeable than sodium iodide.





Selectivity Sequences for the Metal Cation Series

The selectivity sequences corresponding to the metal cation series were also measured for the iodides, formates, salicylates and acetates (Table 3), in some lipid systems. A summary of the sequence obtained is shown in Table 4. It can be seen from this last table that the selectivities obtained depend on the anion accompanying the movement of the cation and, in some cases, on the lipid system used. Thus, for anions such as formate and salicylate, the sequence for the cation selectivity obtained is independent of the lipid system, whereas for acetate and iodide it is dependent on the lipid composition of the liposomes.

It can be observed in Table 4 that the only selectivity sequence for cations that does not coincide with any of the eleven Eisenman sequences (Eisenman, 1961) is that corresponding to acetate salts in the PC+cholesterol system.

The Effect of Gramicidin A on the Rate of Leakage of Acetic and Formic Acid across Liposomes below their Transition Temperature

Isotope leakage experiments across dipalmitoyl-lecithin liposomes below their transition temperature were also carried out, using simultaneously the C^{14} and H^3 isotopes of formic and acetic acid. The results shown in Table 5 indicate that the increase in the permeability to formic acid, induced by gramicidin A, is much greater than acetic acid. However, if gramicidin A is not added to the lipid system, the rate of leakage of acetic acid is greater than formic acid.

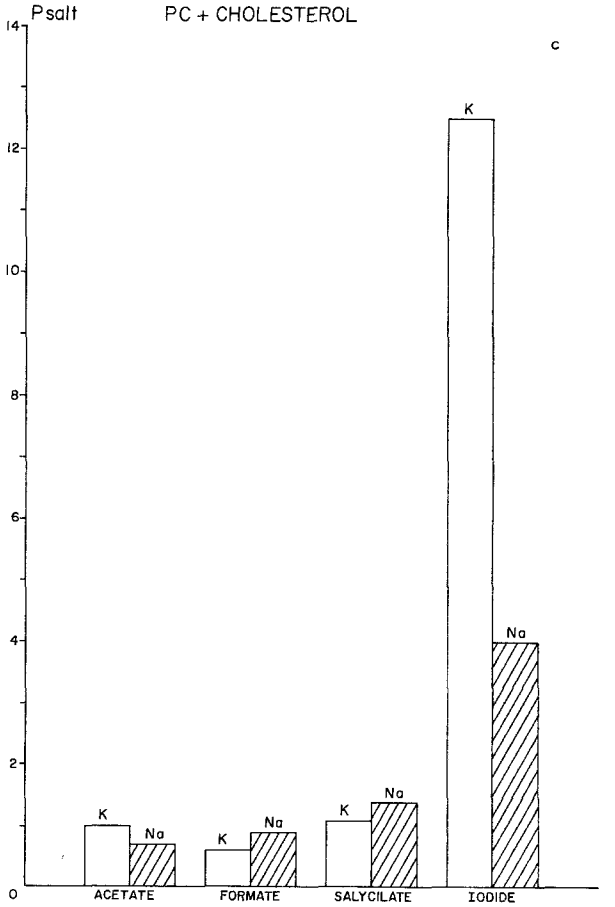


Fig. 3. Relative permeabilities of acetate, salicylate, formate and iodide salts of sodium and potassium across gramicidin-containing liposomes of different lipid composition. (*a*): DPL liposomes; (*b*): PC liposomes; (*c*): PC+48% cholesterol liposomes. $P_{K-Ac}=1$

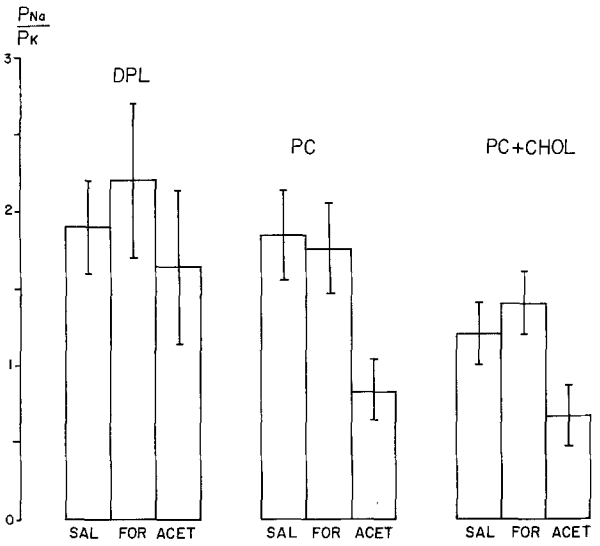


Fig. 4. Permeability ratios (P_{Na}/P_K) for acetate, formate and salicylate salts in liposomes of different lipid composition

Table 4. Cation selectivity sequences for different anions

DPL liposomes		Sequence[a]
Formates	Na > K > Rb > Cs > Li	VII
Salicylates	Na > Li > K	X
Acetates	Li > Na > K	XI
Iodates	K ≃ Na	–
PC liposomes		
Formates	Na > K > Rb > Cs > Li	VII
Salicylates	Na > Li > K	X
Acetates	K > Na > Li	I → VI
Iodates	K ≃ Na	–
PC+48% Cholesterol liposomes		
Formates	Na > K	–
Salicylates	Na > Li > K	X
Acetates	K > Li > Na	?
Iodates	Cs > Rb > K > Na > Li	I

[a] According to Eisenman (1961).

Table 3. Permeability ratios (P_{C+}/P_{Li+}) from salts formed by different anions in liposomes of different lipid compositions[a]

Salt	DPL	PC	PC+Chol
CsI:LiI			5.9
RbI:LiI			4.1
KI:LiI			3.4
NaI:LiI			1.1
K–Sal:Li–Sal	0.82	1.0	0.80
Na–Sal:Li–Sal	1.66	1.2	1.00
Na–For:Li–For	3.8	10.0	
K–For:Li–For	2.2	4.3	
Rb–For:Li–For	1.8	4.2	
Cs–For:Li–For	1.3	3.8	
K–Ac:Li–Ac	0.22	1.96	1.23
Na–Ac:Li–Ac	0.72	1.60	0.82

[a] Temp.: 30 °C. Gramicidin concentration: 5 μg/μmol lipid. Salt concentration: 0.1 M.

Table 5. The rate of leakage of C^{14} and H^3 labeled acetic and formic acid across DPL liposomes containing gramicidin A[a]

Isotope	% Isotope leakage after 30′	
	No Gramicidin A	Gramicidin A
a) C^{14}-acetic acid	14.0	22.9
H^3-formic acid	6.3	35.1
b) H^3-acetic acid	16.3	19.2
C^{14}-formic acid	6.6	30.9

[a] Temp. = 20 °C.

Discussion

The Effect of Gramicidin A on the Volume Changes of Liposomes below Their Transition Temperature

Several authors have reported previously (Goodall, 1971; Hladky & Haydon, 1972) that membrane thickness is an important parameter to consider in the conductance induced by gramicidin A at high concentration in black lipid membranes. This parameter has also been bound to influence the water permeability induced in liposomes by gramicidin A (Boehler, De Gier & Van Deenen, 1978).

According to Haydon and Hladky (1972), the origin of this effect lies in the observation that for decreasing thickness both the frequency of occurrence and the duration of the channels formed by gramicidin increases. Thus, the equilibrium between conducting and nonconducting units shifts in favor of the former in thinner membranes.

The results reported in Fig. 2 also indicate clearly that the effect that gramicidin has on the equilibrium volume changes of liposomes is much greater in the dimyristoyl-lecithin membrane system than in any of the saturated lecithin liposomes with longer hydrocarbon chains. The thickness of the hydrocarbon part of DML membranes at 23 °C is 2.5–2.6 mm (Oldfield, Meadows, Rice & Jacobs, 1978), a value that is very close to the length of one of the dimeric structures proposed by Urry (Urry et al., 1971), the $\Pi^6_{L,D}$ helix, as a model for the gramicidin channel.

Salt Permeability Induced by Gramicidin A

The present work has shown that the gramicidin-induced osmotic changes of liposomes suspended in

different salts markedly depend on the nature of both anion and liposome membrane.

It is a well known fact that liposomes are impermeable to cations (Bangham, Standish & Watkins, 1965), so that in order to induce the permeability to salts, it is necessary to add ionophores that increase cation permeability (Henderson et al., 1969). When gramicidin A is present the cation movement occurs via the gramicidin channel, but the movement of the anion can occur by at least three mechanisms: (i) diffusion by the lipid phase of the membrane, (ii) the undissociated acid moves by the lipid phase followed by cation-proton exchange across the gramicidin channel, (iii) anion and cation may permeate simultaneously through the gramicidin channel.

Though it is evident that the above mechanisms can occur simultaneously, the extent by which any of them may predominate will mainly depend on the relative permeability of the lipid phase to different anions in relation to that of the gramicidin channel.

The order of permeation of acetate and formate salts in PC and PC+cholesterol liposomes (acetate salts > formate salts) coincides with the order expected from the activities in solution of the undissociated acid molecules (Table 6), thus indicating the small contribution to the selectivity of the diffusion process in liposomes above their transition temperature. By contrast, in DPL liposomes below their transition temperature, formate salts are more permeable than acetate salts (Table 2).

The effect that gramicidin has on the anion permeability of liposomes was also investigated by using an independent method (Table 5). The results shown in this Table also indicate that gramicidin induces a greater permeability to formic acid than to acetic acid.

The greater permeability of the different types of liposomes used to salycilate than to acetate or formate salts (Fig. 3) is not an unexpected finding, since this former molecule has a very high partition coefficient in a hydrophobic phase (Table 6). However, a similar permeability sequence for salycilate salts is also observed below the transition temperature (Fig. 3a), where the contribution of partitioning in the lipid phase is considerably reduced due to the solid-crystalline nature of the hydrocarbon chains.

A factor that may contribute to the observed magnitude of the volume changes in the presence of salycilate salts is the adsorption of this ion in lipid membranes (Singer & Bangham, 1971; McLaughlin, 1973). Such a selective adsorption increases the negative surface charge of liposomes, thus leading to an increase in the interlamellar distance of such structures (Singer & Bangham, 1971). As a consequence of this effect, part of the volume changes observed in the presence of salycilate salts cannot be considered to be due to the permeability of the salt.

Table 6. Some physicochemical parameters of weak acids

Acid	pK_a[a]	Partition coefficient[b]	
		$K_{butanol}$	K_{ether}
Formic acid	3.77	0.85	0.42
Acetic acid	4.76	1.2	0.52
Salicyclic acid	2.94	117.0[c]	236.0[c]

[a] Taken from Hendrikson, Cram and Hammond (1970).
[b] Taken from Collander (1950).
[c] The distribution of the undissociated molecules has been calculated, assuming that only undissociated molecules are soluble in the organic phase.

The effect that incorporation of cholesterol in PC liposomes has on the permeability of iodide salts (Fig. 3*c*) is similar to that reported previously by Szabo, Eisenman, McLaughlin & Krasne, 1972). These authors have shown that cholesterol produces a 100-fold increase in iodide conductance of black lipid membranes. They have argued that such an effect is consistent with the appearance of a positive electrical potential within the membrane, possibly due to changes in dipole orientation, or, alternatively, it is possibly the existence of specific interactions between polyiodides and the cholesterol molecule.

In fact, the permeability of cholesterol-containing liposomes to iodide salts is further increased when the external solution is saturated with I_2 (Table 2), indicating that iodide permeation is facilitated by the formation of a polyiodide complex between iodide and molecular I_2 that is more permeable (Finkelstein & Cass, 1968; McLaughlin, Szabo, Eisenman and Ciani, 1970).

Cation Selectivity of the Gramicidin Channel in the Presence of Acetate and Formate Salts

Acetate salts were the only weak acid salts used in this work that exhibited different sequences of cation selectivity in liposomes of various lipid composition. Thus, in DPL liposomes, the cation selectivity obtained is greater (sequence XI) than in PC and PC+cholesterol liposomes (Table 4).

This differential behavior of acetate salts can be explained simply by assuming a greater contribution of the gramicidin channel to the permeability of acetate in liposomes that are below as compared to above the transition temperature.

As it was discussed before (Cohen, 1975*a*), the sequence in which different solutes seem to be permeating across the gramicidin channel follows their molar volumes rather than their cylindrical radius.

Table 7. Permeability ratios (P_C+/P_K+) for different membranes containing gramicidin A

Author	P_{Cs}/P_K	P_{Rb}/P_K	P_{Na}/P_K	P_{Li}/P_K
Myers and Haydon[a] (1972)	1.79	1.38	0.32	0.10
Myers and Haydon[b] (1972)	1.33	1.04	0.29	0.08
Eisenman et al. (1978)	1.18	1.07	0.33	–
This work	1.73	1.20	0.32	0.29

[a] From bi-ionic potential of membranes formed by lecithin + cholesterol + decane.
[b] From bi-ionic potential of membranes formed by glyceryl monooleate + decane.

The partial molar volume reported for acetate of 41.5 ml (Monk, 1961) is very similar to the value which can be calculated for formamide (40.0 ml), indicating that acetate permeation across the channel would be consistent with previous data on the nonelectrolyte selectivity (Cohen, 1975*a*).

Furthermore, the present finding that the permeability of formamidine acetate across DPL liposomes is even greater than potassium or sodium salts (Table 2) suggests that the presence of organic cations inside the channel would further reduce the electrostatic barrier for anion movement. This selective permeability of the gramicidin channel to formamidine has been reported previously by Eisenman, Krasne and Ciani (1976).

Eisenman et al. (1976) have also shown that the permeability of acetamidine across the gramicidin channel is very small, a result which agrees with the observed impermeability of acetamidine acetate across DPL liposomes (Table 2).

The presence of acetate ions inside the gramicidin channel may modify the normal cation selectivity of this structure, by acting like binding sites for cations. Thus, the observed sequence XI for cation selectivity follows the order expected for binding to a high field strength anion in Eisenman's theory of ion exchange equilibria.

In effect, the activity coefficients of acetate salts in aqueous solutions increase from lithium to cesium (Robinson & Stokes, 1970). The stability sequences for acetate salts in aqueous solutions also agree with the sequence XI of cation selectivity, the lithium acetate association constant being 2.75 times greater than sodium (Archer & Monk, 1964).

Similar considerations can also be applied to formate permeation across the gramicidin channel, since carboxylic groups of acids with a pK_a less than acetic acid (Table 6) will also determine such cation selectivity.

In the case of formate salts, the sequence VII of cation selectivity was obtained (Table 4). Such a result indicates that the contribution of the anion mobility across the channel may be important in determining the observed cation selectivities.

A higher permeability of the gramicidin channel to formate compared to acetate would also explain the observed difference between the sequences for cation selectivity of formate salts and acetate salts in liposomes above their transition temperature. The cation selectivity exhibited by acetate salts can be explained by a predominance in such liposomes of acetate permeation by the undissociated acid mechanism (Alger & Prestegard, 1979). This is not surprising since acetic acid is a weaker acid than formic acid and so the flux of undissociated acid molecules across the lipid phase of the membrane will also be greater.

The cation selectivity sequences obtained in the presence of acetate salts across PC and PC + cholesterol liposomes have indicated that the potassium permeability is greater than the sodium permeability (Table 4), as if such a selectivity were determined by cation permeation across the channel. In effect, the cation selectivity of the gramicidin channel coincides with Eisenman sequence I (Myers & Haydon, 1972; Eisenman et al., 1976).

In order to understand the origin of this selectivity, it is important to note that a necessary step in the process of salt permeation by the undissociated acid mechanism is the cation-proton exchange across the gramicidin channel (Henderson et al., 1969; Singer & Bangham, 1971).

At the relatively high concentration of gramicidin used in this work, no substantial limitations of the permeation process would be expected to originate from the cation-proton exchange across the channel, especially since the proton permeability induced by gramicidin is much higher than for any of the other monoatomic monovalent cations (Hladky & Haydon, 1972; Myers & Haydon, 1972).

The fact that the magnitude of the cation selectivities obtained in the presence of acetate salts in PC and PC + cholesterol liposomes (Table 3) is much smaller than those determined for the gramicidin channel (*see* Table 7) may be simply due to the presence of a very small concentration of protons inside the liposomes compared with the much greater cation concentration in the external aqueous solution. However, the contribution of acetate permeation across the gramicidin channel can also lead to this apparent loss of selectivity.

Cation Selectivity of the Gramicidin Channel in the Presence of Iodide and Salycilate Salts

The mobility of iodide across the lipid phase of liposomes appear to be the controlling factor in determin-

ing the sequences of cation selectivity obtained from iodide salts permeation. Thus, when iodide permeability is small, as in DPL and PC liposomes (Table 2), no cation selectivity could be measured. On the other hand, as a consequence of the increased iodide permeability in membranes with cholesterol, anion movement is no longer the rate-limiting step of the permeation process. In this case, the sequence of the cation selectivity would be expected to agree with sequence I.

The values reported in Table 3 for the cation selectivity of iodide salts permeating across cholesterol-containing liposomes are in very good agreement with the cation selectivity of the gramicidin channel obtained from the measurement of biionic potential in black lipid membranes (Table 7). However, since the permeability ratios shown in this Table are concentration dependent, a proper comparison should take into account the salt concentration used in such determinations. At the concentration employed in the present work (100 mM), Urban et al. (1980) have recently calculated that the K/Na permeability ratio is about 3:1, a value that is identical to the present value obtained from iodide salt permeabilities.

Salycilate, like iodide, can also permeate as an anion across the lipid phase of liposome membranes (Singer, 1973). However, unlike iodide salts, salycilates do not yield a sequence I of cation selectivity in any of the liposomes investigated. Instead, a higher sequence of cation selectivity (sequence X) was obtained in all liposomes investigated (Table 4).

Such a sequence cannot be explained by assuming different membrane pathways for anion movement, since it is very unlikely that salycilate can permeate through the gramicidin channel, due to steric reasons. However, the presence of salycilate at the membrane surface may lead to changes in the normal cation selectivity of the gramicidin channel.

Other Factors Possibly Involved in Determining Salt Permeation across Gramicidin-Containing Liposomes

The previous analysis of the various mechanisms that can be involved in salt permeation across gramicidin-containing liposomes is based on several assumptions, one of them being that the lipids themselves do not exert specific effects on the permeability properties and structure of the gramicidin channel.

There are, however, some indications that the permeability of the gramicidin channel to some solutes is different when it is present in liposomes of different lipid composition. Thus, in dimyristoyl-lecithin liposomes (DML), the ratio of the permeability of acetamide to urea is smaller than in DPL, in spite of the much lower temperature at which the measurements in DML were carried out (Cohen, 1975*b*, Table 5). In agreement with this data, it was found that the activation energy for permeation of urea across DML liposomes is smaller than for acetamide, suggesting that the gramicidin channel, in such membranes, is permeable to urea.

The main difference between the various types of liposomes used in this work is the different degrees of fluidity of the hydrocarbon chains of the lecithins. The interaction of gramicidin molecules with such lipids may differ, depending on the fluidity of the hydrocarbon chains. Thus, even though gramicidin does not change the transition temperature of lecithins, it may affect the packing of polar head groups, as indicated by the disappearance of the pretransitional peak in DPL (Chapman, Urbina & Keough, 1974).

Subtle changes in the interaction of the lipid polar head groups with the gramicidin molecules, induced by changes in the packing of the polar head groups, may lead to changes in structure or permeability of the channel. The presence of some anions at the membrane surface, i.e., salycilate, may have similar effects.

I am grateful to Prof. P.C. Crogham for critically reading this manuscript. This work was supported by C.D.C.H. (Universidad Central de Venezuela) and CONICIT (Grant No. 31.26.S1-0532).

References

Alger, J.R., Prestegard, J.H. 1979. Nuclear magnetic resonance study of acetic acid permeation of large unilamellar vesicle membranes. *Biophys. J.* **28**:1–14

Archer, D.W., Monk, C.B. 1964. Ion-association of some acetates by pH (glass-electrode) measurements. *J. Chem. Soc.* **(1964)**:3117–3122

Bamberg, E., Apell, H.J., Alpes, H. 1977. Structure of the gramicidin A channel: Discrimination between the $\Pi_{L,D}$ helix and the β helix by electrical measurements with lipid bilayer membranes. *Proc. Natl. Acad. Sci. USA* **74**:2402–2406

Bangham, A.D., Hill, M.W., Miller, N. 1974. Preparation and use of liposomes as models of biological membranes. *In:* Methods in Membrane Biology. Vol. 1, pp. 1–68. E.D. Korn, editor. Plenum Press, New York

Bangham, A.D., Standish, M., Watkins, J.C. 1965. Diffusion of univalent ions across the lamellae of swollen phospholipids. *J. Mol. Biol.* **13**:238–252

Boehler, B.A., De Gier, J., Van Deenen, L.L.M. 1978. The effect of gramicidin A on the temperature dependence of water permeation through liposomal membranes prepared from phosphatidyl-cholines with different chain lengths. *Biochim. Biophys. Acta* **512**:480–488

Bray, G.A. 1960. A simple efficient liquid scintillator for counting aqueous solutions in a liquid scintillation counter. *Anal. Biochem.* **1**:279–285

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

Chappell, J.B., Crofts, A.R. 1965. Gramicidin and ion transport in isolated liver mitochondria. *Biochem. J.* **95**:393–402

Cohen, B.E. 1975*a*. The permeability of liposomes to nonelectrolytes: I. Activation energies for permeation. *J. Membrane Biol.* **20**:205–234

Cohen, B.E. 1975*b*. The permeability of liposomes to nonelectrolytes: II. The effect of nystatin and gramicidin A. *J. Membrane Biol.* **20**:235–268

Cohen, B.E., Bangham, A.D. 1972. Diffusion of small nonelectrolytes across liposomes membranes. *Nature (London)* **236**:173–174

Collander, R. 1950. The distribution of organic compounds between isobutanol and water. *Acta Chem. Scand* **4**:1085–1098

Eisenman, G. 1961. On the elementary atomic origin of equilibrium ionic specificity. *In:* Symposium on Membrane Transport and Metabolism. A. Kleinzeller and A. Kotyk, editor pp. 163–179. Academic Press, New York

Eisenman, G., Krasne, S., Ciani, S. 1976. Further studies on ion selectivity. *In:* Ion and Enzyme Electrodes in Biology and Medicine. pp. 3–21. M. Kessler, L. Clark, D. Lubbers, I. Silver, and W. Simon, editor. Urban & Schwarzenberg, Munich

Eisenman, G., Sandblom, J., Neher, E. 1977. Ionic selectivity, saturation, binding and block in the gramicidin A channel: A preliminary report. *In:* Metal-ligand Interaction in Organic Chemistry and Biochemistry. B. Pullman and N. Goldblum, editors. Part 2, pp. 1–36. D. Reidel, Dordrecht-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**:307–346

Finkelstein, A., Cass, A. 1968. Permeability and electric properties of thin lipid membranes. *J. Gen. Physiol.* **52**:145*s*–172*s*

Goodall, M.C. 1971. Thickness dependence in the action of gramicidin A on lipid bilayers. *Arch. Biochim. Biophys.* **147**:129–135

Haydon, D.A., Hladky, S.B. 1972. Ion transport across thin lipid membranes: A critical discussion of mechanism in selected systems. *Q. Rev. Biophys.* **5**:187–282

Henderson, P.J.F., McGivan, J.D., Chappell, J.B. 1969. The action of certain antibiotics on mitochondrial, erythrocyte and artificial phospholipid membranes. *Biochem. J.* **111**:521–535

Hendrikson, J.B., Cram, D.J., Hammond, G.S. 1970. Organic Chemistry. McGraw-Hill, New York

Hill, M.W., Cohen, B.E. 1972. A simple method of determining relative permeabilities of liposomes to nonelectrolytes. *Biochim. Biophys. Acta* **290**:403–407

Hladky, S.B. 1972. The two site lattice model for the pore. Appendix B′. Ph.D. dissertation, Cambridge University, England

Hladky, S.B., Haydon, D.A. 1970. Discreteness of conductance changes in bimolecular lipid membranes in the presence of certain antibiotics. *Nature (London)* **255**:451–453

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

Krasne, S., Eisenman, G., Szabo, G. 1971. Freezing and melting of lipid bilayers and the mode of action of nonactin, valinomycin and gramicidin. *Science* **174**:412–415

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

McLaughlin, S. 1973. Salicylates and phospholipid bilayer membranes. *Nature (London)* **243**:234–236

McLaughlin, S.G.A., Szabo, G., Eisenman, G., Ciani, S.M. 1970. Surface charge and the conductance of phospholipid membranes. *Proc. Natl. Acad. Sci. USA* **67**:1268–1275

Monk, C.B. 1961. Electrolytic dissociation. P. 271. Academic Press, New York

Mueller, P., Rudin, D.O. 1967. Development of $K^+ - Na^+$ discrimination in experimental bimolecular lipid membranes by macrocyclic antibiotics. *Biochem. Biophys. Res. Commun.* **26**:398–404

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

Oldfield, E., Meadows, M., Rice, D., Jacobs, R. 1978. Spectroscopic studies of specifically deuterium labeled membrane systems. Nuclear magnetic resonance investigation of the effects of cholesterol in model systems. *Biochemistry* **17**:2727–2740

Papahadjopoulos, D., Miller, N. 1967. Phospholipid model membranes: I. Structural characteristics of hydrated liquid crystals. *Biochim. Biophys. Acta* **135**:624–638

Pressman, B.C. 1965. Induced active transport of ions into mitochondria. *Proc. Natl. Acad. Sci. USA* **53**:1076–1083

Robinson, R.A., Stokes, R.H. 1970. Electrolyte Solutions. (2nd ed.) Butterworths, London

Rosenberg, P.A., Finkelstein, A. 1978. Water permeability of gramicidin A-treated lipid bilayer membranes. *J. Gen. Physiol.* **72**:341–350

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

Singer, M.A. 1973. Transfer of anions across phospholipid membranes. *Can. J. Physiol. Pharmacol.* **51**:523–531

Singer, M.A., Bangham, A.D. 1971. The consequences of inducing salt permeability in liposomes. *Biochim. Biophys. Acta* **241**:687–692

Szabo, G., Eisenman, G., McLaughlin, S., Krasne, S. 1972. Ionic probes of membrane structures. *Ann. N.Y. Acad. Sci.* **195**:273–295

Tosteson, D.C. 1968. Effect of macrocyclic compounds on the ionic permeability of artificial and natural membranes. *Fed. Proc.* **27**:1269–1277

Urban, B.W., Hladky, S.B., Haydon, D.A. 1980. Ion movements 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 $\Pi_{(L,D)}$ helices. *Proc. Natl. Acad. Sci. USA* **68**:672–676

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

Veatch, W.R., Fossel, E.T., Blout, E.R. 1974. The conformation of gramicidin A. *Biochemistry* **13**:5249–5256

Received 26 June 1981;
revised 10 August 1981 and 5 January 1982