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The Cation-Binding Properties of Gramicidin†

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ABSTRACT: We report studies of the interaction of gramicidin (*ca.* 72% gramicidin A) with K^+ and Cs^+ in methanol using ion selective electrodes, and with K^+ and Ba^{2+} using partitioning of the picrate salts of these ions between methylene chloride containing gramicidin and water. The extraction experiments show that the K^+:gramicidin ratio is 1:1 in methylene chloride and that the extraction equilibrium constant is 3.61×10^{-4} M^{-1}. Gramicidin binds ions very weakly relative to neutral ion carriers such as nonactin and dicyclohexyl-18-crown-6, with the ratio of binding constants being approximately 10^{-6}. Ion transport experiments using U-tubes containing gramicidin in chloroform or methylene chloride separating a potassium picrate and distilled water solution show that gramicidin transports ions through these long membranes much more poorly than diffusional carriers such as dicyclohexyl-18-crown-6. Since membranes containing gramicidin conduct ions as well as those containing diffusional carriers, gramicidin acts by a different mechanism, probably as a channel mediator as suggested in the literature. Our experiments appear to rule out the possibility that gramicidin can act as both a channel and a diffusional carrier. The poor ion complexing ability of gramicidin relative to neutral carriers is suggested to be due to several factors including looseness of fit of ions in the gramicidin channel, and possibly the linearity of gramicidin molecule.

Ion transporting antibiotics fall into two categories: (1) the diffusional carriers which act by diffusing through the membrane with the ion complexed in the central cavity (*e.g.*, nonactin, valinomycin, and nigericin); and (2) the channel carriers which act by forming channels or pores through the membrane (*e.g.*, gramicidin). The diffusional carriers can further be subdivided into the categories of neutral carriers and carboxylic acid carriers (Pressman, 1968). The neutral carriers (*e.g.*, valinomycin, nonactin, and crown ethers) are cyclic compounds with a high proportion of oxygen. The metal complex of the neutral carriers has a net positive charge which is insulated from the membrane by a nonpolar outer coat. The carboxylic acid diffusional carriers, on the other hand, are usually linear compounds which also contain a high proportion of oxygen (*e.g.*, X-537a, nigericin, and monesin). The metal complex of these antibiotics has no net charge.

The channel carriers have been distinguished from the diffusional carriers by measuring the conductance of artificial membranes in the liquid and frozen states (Krasne *et al.*, 1971) and by studies of the conductivity of artificial membranes (Myers and Haydon, 1972; Hladky and Haydon, 1972). The conductivity of lipid bilayers in the liquid state containing 10^{-7} M valinomycin or 10^{-9} M gramicidin is about 10^{-4} ohm^{-1} cm^{-2}. Upon freezing, the conductivity of membranes containing valinomycin drops to about 10^{-9} ohm^{-1} cm^{-2} but the conductivity of membranes containing gramicidin does not change appreciably (Krasne *et al.*, 1971).

The conformations of neutral diffusional carriers such as valinomycin have been studied in solution in the presence and absence of cations by nuclear magnetic resonance (nmr), infrared spectroscopy (ir), optical rotary dispersion (ORD), and circular dichroism (CD) (Patel and Tonelli, 1973; Ivanov *et al.*, 1969; Ohnishi and Urry, 1969). Crystal structures of uncomplexed and the K^+ complex of valinomycin have been reported (Pinkerton *et al.*, 1969; Duax *et al.*, 1972). Crystal structures of cation complexes of several other neutral carriers including nonactin (Kilbourn *et*

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al., 1967) and several crown ethers have also been reported (see, for example, Mercer and Truter, 1973a,b). The spectral and solid state data on valinomycin and other neutral diffusional carriers show that these compounds complex ions by orienting polar functionalities toward the central cavity leaving a nonpolar coat on the surface. The size of the central cavity and the field strength of the ligands account for the ion selectivity of these agents (Diebler *et al.*, 1969; Eisenman, 1969).

The ion selectivity and crystal structure of several carboxylic acid diffusional carriers have also been studied (Johnson *et al.*, 1970; Ashton and Steinrauf, 1970; Pressman, 1968). These compounds also show substantial ion selectivity even though they are linear. As with the neutral diffusional carriers this selectivity is probably due to both the size of the central cavity and the field strength of the ligands.

Gramicidin A is the channel mediator which has received the most experimental attention. Gramicidin A is a linear pentadecapeptide containing alternating D and L residues as shown. Gramicidin B has the L-Trp in position 11 replaced

HCO-L-Val-D-Gly-L-Ala-D-Leu-L-Ala-D-Val-L-Val-D-Val-L-Trp-D-Leu-L-Trp-D-Leu-L-Trp-D-Leu-L-Trp-$NHCH_2CH_2OH$ [11]

with L-Phe and gramicidin C has the L-Trp in position 11 replaced with L-Tyr. Commercially available gramicidin contains about 72% gramicidin A and 28% gramicidins B and C (Glickson *et al.*, 1972). The conformation of gramicidin A in organic solvents is solvent dependent based on studies in the absence of ions using nmr, ORD, and CD (Glickson *et al.*, 1972; Urry *et al.*, 1972; Isbell *et al.*, 1972). However, nmr experiments suggest that gramicidin A forms a new type of left-handed helix which is 15 Å long and that in membranes two gramicidin A helices dimerize to form a 30-Å channel which transports ions (Urry, 1972; Urry *et al.*, 1971). Studies on the rate of channel formation support the proposal that gramicidin dimerizes to form conducting channels (Bamberg and Lauger, 1973; Hladky and Haydon, 1972; Myers and Haydon, 1972). Gramicidin shows the following selectivity sequence for the transport of univalent cations through artificial membranes: $H^+ > NH_4^+ > Cs^+ > Rb^+ > K^+ > Na^+ > Li^+$. It does not significantly transport divalent cations such as Ca^{2+} through artificial membranes (Myers and Haydon, 1972; Hladky and Haydon, 1972). However, the differences in ease of transport of Na^+, K^+, Rb^+, and Cs^+ through the membrane are much smaller than the corresponding differences for crown ethers or nonactin (Eisenman *et al.*, 1968).

The ion-binding and transport properties of gramicidin, which are reported in this paper, are of importance since the structure and function of this antibiotic are different from the neutral and carboxylic acid diffusional carriers. Gramicidin is linear and contains no carboxylic acid group and, of course, is a channel forming antibiotic.

Our studies suggest that U-tube experiments and studies of ion-binding properties can provide new ways of distinguishing diffusional carriers from channel forming materials and provide information on the structural requirements for ion binding in antibiotics.

Experimental Section

Materials. All solvents were spectral grade and doubly distilled deionized water was used. The salts, KCl, KOH, CsCl, and $Ba(OH)_2$, were analytical reagent grade and were used without further purification. Tetraethylammonium chloride was purchased from Aldrich Chemical Co. and dried before use. Gramicidin was used as purchased from Nutritional Biochemical Corporation (*ca.* 72% gramicidin A, 9% gramicidin B, and 19% gramicidin C); the mp of gramicidin was 230–232°. It should be noted that the per cent of gramicidins A, B, and C in commercial preparations is variable (Hunter and Schwartz, 1967). Dicyclohexyl-18-crown-6, technical grade, was used as purchased from Aldrich Chemical Co. This crown ether was a brown viscous liquid and experiments discussed below indicate its purity was less than 100%. Because this material was used only to verify our procedures further purification was not deemed necessary.

All ultraviolet and visible spectra were measured using a Cary 17 and all measurements were made at room temperature.

Solubility and Partition Coefficients. The solubility of gramicidin was estimated by placing a known weight of gramicidin in an erlenmeyer flask and portions of solvent were added until gramicidin had completely dissolved. The solubilities estimated (±20%) in this way were: H_2O, 44.0 mg/2300 ml (1.04×10^{-5} M), and CH_2Cl_2, 4.1 mg/9 ml (2.47×10^{-4} M).[1] An upper limit to the partition coefficient of gramicidin between H_2O and CH_2Cl_2 and H_2O and $CHCl_3$ was found by shaking 10 ml of a solution of gramicidin in the organic solvent (4.6 mg/ml of CH_2Cl_2 and 3.5 mg/ml of $CHCl_3$) with 10 ml of water. The uv spectra of the water layer showed no absorbance in the range 250–350 mμ, using $E_{1\,cm}^{1\%}$ at 281.5 mμ of 125 (Tishler *et al.*, 1941). Assuming a maximum absorbance of 0.001, an upper limit to the partition coefficient can be estimated: CH_2Cl_2, 1.76×10^{-4}; $CHCl_3$, 2.27×10^{-4}.

Ion Selective Electrodes. A Corning monovalent cation glass electrode (Catalog No. 476220) of the NAS 27-4 type described by Eisenman (Eisenman *et al.*, 1957; Eisenman, 1962) and a silver|silver chloride reference electrode were used. An Orion Model 407 specific ion meter (accuracy ±0.3 mV) was used. The Ag|AgCl electrodes were prepared by flash coating no. 14 gauge silver wire in 0.1 N HCl for about 15 sec at 50 mA according to the directions of Frensdorf (1971a). All measurements were made using at least five different Ag|AgCl reference electrodes and were made after the meter stabilized. All measurements were made at a Cl^- concentration of 10^{-2} M with the $[Cl^-]$ being maintained at this value with the proper amount of $Et_4N^+Cl^-$. Measurements using KCl stock solution indicated that three reference electrodes consistently gave almost identical results (within ±1.5 mV) and these were used for all subsequent measurements. The dissociation constant of KCl in methanol of 10 l./mol (Sillen and Martell, 1964) was used to calculate the K^+ concentration in 0.01, 0.001, and 0.0001 M KCl; the dissociation constant of CsCl in methanol was not available, as far as is known, but was assumed to be 10 l./mol. The change in emf upon going from 0.01 to 0.001 M KCl was 62.4 and from 0.001 to 0.0001 was 48.8 and the change in emf upon going from 10^{-4} to 10^{-5} M solutions was 40.1 mV. Thus, some deviation from theory is seen at 10^{-4} M. The change in emf upon going from 0.01 M CsCl to 0.001 M was 54.0, while the change upon going from 0.001 to 0.0001 M was 46.0 and the change upon going from 10^{-4} to 10^{-5} M solutions was 33.0. These deviations from theory

[1] A molecular weight of 1837 is assumed for gramicidin for all calculations in this paper.

TABLE I: Summary of Qualitative Extraction Experiments.

Alkali Metal	Composition of Aq Soln: [Alkali Metal] (M)	Composition of Aq Soln: 10^5[Picrate] (M)	Composition of Org Soln: 10^4[Gramicidin] (M)	Solvent	% Extract into Org Layer
Ba^{2+}	0.1146	5.59	2.17	CH_2Cl_2	<0.2
K^+	0.1096	5.63	2.17	CH_2Cl_2	1.0
K^+	8.96×10^{-5}	7.38	2.51	CH_2Cl_2	0.4
K^+	8.96×10^{-5}	7.38	1.93	$CHCl_3$	<0.2

could be due to the assumption that the dissociation constant of CsCl in methanol was 10 l./mol. Addition of solid gramicidin to 10^{-2} M solutions of KCl or CsCl showed no change in emf. A 10^{-5} M solution of KCl with 2.7 mg/ml of gramicidin and a 10^{-5} M solution of CsCl with 3.6 mg/ml of gramicidin had an identical emf reading with 10^{-5} M solutions containing no gramicidin.

Extraction Experiments. Qualitative experiments were performed by shaking 25 ml of organic phase with 25 ml of aqueous phase containing picrate salt for at least 2 min. The absorbance of the organic layer was measured using a 1-cm cell after filtration through a glass wool plug and the per cent extraction was based on an ϵ_{374} of picrate in CH_2Cl_2 and $CHCl_3$ of 1.8×10^4 (Frensdorf, 1971b). The results of these experiments are summarized in Table I.

Because of the small amount of extraction, quantitative extraction experiments were made using 10-cm cells. Fifty milliliters of aqueous picrate solution containing 1.98×10^{-4} M picric acid was vigorously shaken with 50 ml of CH_2Cl_2 containing 2.00×10^{-4} M gramicidin. The CH_2Cl_2 solution was then centrifuged at 8000 rpm for 30 min before the absorbance was measured at 374 mμ. Table II summarizes the results of these experiments.

Transport Experiments. All experiments were performed by placing 7 ml of a $CHCl_3$ or CH_2Cl_2 solution of antibiotic in a U-tube (2 cm i.d., 10 cm long) and placing aqueous picrate salt solution on one side and distilled water on the other side (Pinkerton *et al.*, 1969; Ashton and Steinrauf, 1970). In this configuration the organic solvent was above the bend thus insulating the aqueous solutions from each other. These experiments were run for approximately 96 hr without stirring. In the experiments where the organic layer was stirred, 10 ml of antibiotic solution was used to eliminate the possibility of mechanical transportation. Control experiments with pure $CHCl_3$ or CH_2Cl_2 revealed no transport with these U-tube configurations.

Control Experiments. In order to verify the experimental procedures discussed above extraction, ion selective electrode, and U-tube experiments on solutions containing dicyclohexyl-18-crown-6 were carried out. A CH_2Cl_2 crown ether solution (8.7 mg/100 ml, 2.3×10^{-4} M) was used to extract an equal volume of a KCl–picrate solution (0.104 M KCl, 4.6×10^{-5} M picrate). Under these conditions >99% of the picrate was extracted into the organic phase, while calculations using literature data (Frensdorf, 1971b) indicated the per cent extraction should be about 98.5%.

Measurements of the emf of a 10^{-2} M solution of CsCl in methanol containing 41.6 mg of technical grade dicyclohexyl-18-crown-6 and using the emf data discussed above gave a log K, where $K = [CsCr^+]/[Cs^+][Cr]$, of 3.23. This compares with a reported value of 3.49 for isomer B and 4.61 for isomer A (Frensdorf, 1971a). The reason for this discrepancy is not clear but is probably due to the impurities in the crown ether. For example, if the technical grade crown ether is assumed to be 82% pure then the calculated log K is 3.78.

U-tube experiments using the following configuration (5 ml of distilled H_2O–7 ml of 1.92×10^{-4} M crown ether in $CHCl_3$–5 ml of 8.96×10^{-5} M K^+ and 7.38×10^{-5} M picrate) were run for 95 hr and 50 min without stirring and 119 hr and 20 min with stirring. The equilibrium constants for these experiments are reported in Table V.

Results and Discussion

Tables I and II summarize the qualitative and quantitative extraction experiments on gramicidin. The extent of extraction of the alkali metal was indirectly measured by measuring the amount of picrate anion extracted into the

TABLE II: Summary of Quantitative Extraction Experiments on Gramicidin Solutions in Methylene Chloride Shaken with Aqueous Potassium Hydroxide–Picric Acid Solutions.

C_{K^+}	$A_{374\ m\mu}$*	$10^6 C_{Pic}^{Tot}$*[a]	$\gamma_{K^+}\gamma_{Pic^-}$[b]	$10^4 C_{Pic}$	$10^4 C_{Gr}$*	$10^4(C_{K^+} C_{Pic} C_{Gr}{}^* \gamma_{K^+}\gamma_{Pic})^{1/2}$
0.911	0.466	2.58	0.59	1.96	1.97	1.43
0.729	0.462	2.57	0.55	1.96	1.97	1.24
0.547	0.419	2.33	0.53	1.96	1.98	1.06
0.365	0.366	2.03	0.54	1.96	1.98	0.875
0.182	0.274	1.52	0.58	1.97	1.99	0.643
0.911	0.351	1.95	0.58	1.96	0.981	1.01
0.911	0.248	1.38	0.58	1.97	0.486	0.711

[a] Based on an ϵ_b of 1.8×10^5 (Frensdorf 1971a). [b] Activity coefficients calculated according to the method of Eisenman *et al.* (1969), using Table 12-2-7A of Harned and Owen (1957).

organic phase using visible spectroscopy. The "soft" picrate ion was extracted into the organic phase to maintain charge balance much more readily than "hard" ions such as chloride or hydroxide.

The extraction of ions into organic layers can be conveniently expressed in terms of several constituent equilibria as shown by Eisenman *et al.* (1969) and by Frensdorf (1971b). The extraction equilibrium constant K_i describes the extraction process for 1:1 stoichiometry, where the asterisk indicates that the species is in the organic phase.

$$K^+ + Pic + Gr^* \underset{K_i}{\rightleftharpoons} KGr^{+*} + Pic^* \quad (1)$$

The ion pairing process in the organic phase has the equilibrium constant K_{isx}.

$$KGr^{+*} + Pic^* \underset{K_{isx}}{\rightleftharpoons} KGrPic^* \quad (2)$$

A simplification is obtained in the case of gramicidin since the gramicidin is not significantly partitioned into the aqueous phase. For CH_2Cl_2 and $CHCl_3$ the upper limits for the partition coefficient P_e are 1.76×10^{-4} and 2.27×10^{-4}, respectively.

$$Gr^* \overset{Pe}{\rightleftharpoons} Gr \quad (3)$$

A consideration of eq 1, 2, and 3 leads to eq 4 (Eisenman

$$C_{K^+}{}^{Tot*} = C_{Pic}{}^{Tot*} = (K_i C_{K^+} C_{Pic} C_{Gr}{}^* y_{K^+} y_{Pic})^{1/2} + K_{isx}(K_i C_{K^+} C_{Pic} C_{Gr}{}^* y_{K^+} y_{Pic}) \quad (4)$$

et al., 1969). This equation can be further simplified to eq 5

$$C_{K^+}{}^{Tot*} = C_{Pic}{}^{Tot*} = (K_i C_{K^+} C_{Pic} C_{Gr}{}^* y_{K^+} y_{Pic})^{1/2} \quad (5)$$

if ion pairing is unimportant ($K_{isx} \sim 0$).[2]

Eisenman *et al.* (1969) found that K_{isx} was negligible in methylene chloride in their extraction studies of macrolide actin antibiotics. Using a K_{isx} of 2.7×10^4 observed for the K^+–dicyclohexyl-18-crown-6 complex (Frensdorf, 1971b) one estimates that the ion pairing is less than 2% at $C_{K^+}{}^{Tot*} = C_{Pic}{}^{Tot*} = 5 \times 10^{-6}$.

Figure 1 shows a plot of eq 5. The linearity of this plot substantiates the assumption that K_{isx} was negligible.

The plot of $C_{Pic}{}^{Tot*}$ *vs.* $(C_{K^+} C_{Pic} C_{Gr}{}^* y_{K^+} y_{Pic})^{1/2}$ when only $C_{Gr}{}^*$ is varied can be used to test the 1:1 stoichiometry of the complexation. A consideration of eq 6, 7, and 8 derived by Eisenman *et al.* (1969) indicates that this plot would not be linear in the cases of 1:2, 2:1, and 2:2 K^+:Gr complexing.

$$nK^+ + mGr^* + nPic \underset{}{\overset{K_i(n,m)}{\rightleftharpoons}} K_n{}^n Gr_m{}^{+*} + nPic^* \quad (6)$$

$$K_i^{n,m} = \frac{C_{Pic}{}^{*n} C_{K_n{}^+ Gr_m{}^*}}{a_{K^+}{}^n C_{Gr}{}^{*m} a_{Pic}{}^n} \quad (7)$$

$$C_{Pic}{}^{Tot*} = (nK_{K^+}{}^{n,m} a_{K^+}{}^n C_{Gr}{}^{*m} a_{Pic}{}^n)^{1/n+1} \quad (8)$$

Isbell *et al.* (1972) have reported studies of the average molecular weight of gramicidin in dioxane (dielectric constant = 2.21), methanol (dielectric constant = 32.63), and ethyl acetate (dielectric constant = 6.02). They found that in nonpolar solvents such as dioxane, gramicidin was mainly in the dimer state and that in polar solvents such as methanol, gramicidin was mainly in the monomer state. This is not inconsistent with our results of 1:1 K^+:gramicidin stoichiometry. The molecular weight of a 7.62×10^{-4} M gramicidin solution in ethyl acetate was 2080 indicating the presence of only about 12% dimer. Our studies were performed in the more polar solvent methylene chloride (dielectric constant = 9.08) and on a more dilute solution, 2.00×10^{-4} M gramicidin. Under these conditions gramicidin probably exists almost entirely in the monomer state.

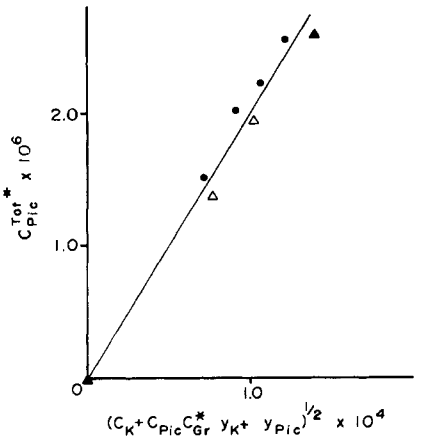

FIGURE 1: (●) Data obtained by varying C_{K^+}; (△) data obtained by varying $C_{Gr}{}^*$. The least-squares slopes, intercepts, and correlation coefficients for the ●, △, and all data are: 1.90×10^{-2}, 1.80×10^{-7}, 0.98; 1.83×10^{-2}, 3.89×10^{-8}, and 1.00; and 1.90×10^{-2}, 1.41×10^{-7}, and 0.98, respectively. The corresponding K_i values are 3.61×10^{-4}, 3.35×10^{-4}, and 3.61×10^{-4} M^{-1}, respectively.

The extraction equilibrium constant $K_i = 3.61 \times 10^{-4}$ M^{-1} for gramicidin in methylene chloride is six orders of magnitude less than the K_i values of nonactin, monactin, dinactin, trinactin, and dicyclohexyl-18-crown-6 which are 190, 850, 2000, 4000, and 160, respectively (Eisenman *et al.*, 1969; Frensdorf, 1971b). Furthermore, this constant drops to approximately zero as the solvent polarity decreases from methylene chloride (dielectric constant = 9.08) to chloroform (dielectric constant = 4.81) (Table I). The relative values of these extraction equilibrium constants are consistent with measurements of the dissociation constants of a GrK^+ or $GrCs^+$ complex in methanol using ion selective electrodes. In these experiments the K^+ and Cs^+ ion concentrations are monitered in the presence and absence of gramicidin, and any complexation would cause a decrease in the measured concentration of the alkali metal ion (Frensdorf, 1971a). No complexing of K^+ or Cs^+ ions by gramicidin was detected in MeOH allowing an upper limit on K of about 1 to be set. The K for dicyclohexyl-18-

$$GrM^+ \overset{K}{\rightleftharpoons} Gr + M^+$$

crown-6-K^+ was 1.023×10^6 for isomer A and 2.399×10^5 for isomer B. The K for the crown-Cs^+ complex was 4.074×10^4 for isomer A and 3.090×10^3 for isomer B (Frensdorf, 1971a). There is evidence that gramicidin is in different conformations in methanol and methylene chloride (Urry *et al.*, 1972) but these differences apparently do not measurably enhance the ion binding ability of gramicidin.

Although our studies were made using only K^+, Cs^+, and Ba^{2+} ions they probably are representative of measurements on other ions. Membrane conductivity data (see below) show that gramicidin renders membranes permeable to only monovalent cations and has little ion selectivity (Myers and Haydon, 1972). Thus, our measurements of complexing and binding constants using K^+ and Cs^+ which

[2]Equation 5 is identical with the $K_e K_d$ expression derived by Frensdorf (1971b) using similar reasoning.

TABLE III: Comparison of the Ion Complexing Data on Crown Ethers[a] and Gramicidin.

Crown Ether	% Ext (K^+)[b]	% Ext (Na^+)[b]	Log $K(K^+)$[c]	Log $K(Na^+)$[c]	Log $K(Cs^+)$[c]
Dicyclohexyl-14-crown-4[d]			1.30	2.18	
Dicyclohexyl-15-crown-5[d]	8.7	19.7	3.58[f]	3.71	2.78[f]
Dicyclohexyl-18-crown-6 (isomer A)[e]	77.8[g]	25.6	6.01	4.08	4.61
Dicyclohexyl-18-crown-6 (isomer B)[e]			5.38	3.68	3.49
Dicyclohexyl-21-crown-7	51.3	22.6	4.30	2.40	4.20
Gramicidin[h]	1.3[h]	*i*	<0[j]		<0[g]

[a] Taken from Frensdorf (1971b) and Pederson (1968). [b] Per cent extraction of M^+ Pic^- into a 7×10^{-5} M methylene chloride solution of crown. The M^+ Pic^- solution was prepared with 0.1 N M^+OH^- and 7×10^{-5} M picric acid. [c] Log of the stability constant ($K = [MCr^+]/[M^+][Cr]$) measured in methanol. [d] The cyclohexyl ring contains a *tert*-butyl substituent. [e] Isomer A is either the D, L, or the meso form of this crown. [f] Evidence of 2:1 complexing. [g] If a 7×10^{-4} M methylene chloride solution of crown is used this value is 99+%. [h] See Table II for conditions. [i] Qualitative experiments involving the addition of NaCl to gramicidin–picrate mixtures did not increase the yellow color of the organic layer. [j] Measured in this paper.

TABLE IV: Ionic Diameters, Pore Sizes, and Cavity Sizes of Interest.[a]

Ion	Ionic Diam (Å)	Compd	Pore or Cavity Size (Å)
Na^+	1.94		
K^+	2.66	14-Crown-4	1.2–1.5
Rb^+	2.94	15-Crown-5	1.7–2.2
Cs^+	3.34	18-Crown-6	2.6–3.2
		21-Crown-7	3.4–4.3
		Gramicidin A ^{4}(L,D)	1.4
		Gramicidin A ^{6}(L,D)	4
		Gramicidin A ^{8}(L,D)	6
		Gramicidin A ^{10}(L,D)	8

[a] All data taken from Frensdorf (1971a) except for that for gramicidin which was taken from Urry *et al.* (1971).

show that gramicidin has little ion complexing ability should hold for other ions such as Rb^+ which were not included in our studies.

The ratios of ion permeabilities of membranes containing gramicidin relative to Na^+ are 3.1, 4.3, and 5.6 for K^+, Rb^+, and Cs^+ (Myers and Haydon, 1972). This lack of transport selectivity rules out the explanation that gramicidin binds K^+ and Cs^+ poorly because it has a pore size and ion binding properties similar to 14-crown-4 and is therefore in a π^4(L,D) helix (see Tables III and IV for a comparison of ion binding properties and pore sizes). The diameter of a π^4(L,D) helix, 1.4 Å, is much too small to transport Cs^+ ions through membranes or lipid bilayers.

Eisenman *et al.* (1968) derived eq 9 relating extraction equilibria (K_i) to membrane conductance (G) where u^* is

$$\frac{K_{ij}}{K_{ij}} = \frac{G_0(\mathrm{I})}{G_0(\mathrm{J})} \frac{u_{js}^*}{u_{is}^*} \qquad (9)$$

the mobility of the charged complexes in the membrane. This equation has been used mainly to relate ratios of extraction equilibrium constants (K_i) to ratios of membrane conductances where the neutral carrier is the same and the ion is varied. This equation successfully predicts the conductivity ratios of macrolide actin antibiotics from the ratios of extraction equilibrium constants. A modified version of eq 9 successfully predicts the conductivity ratios of crown ethers from the ratios of extraction equilibrium constants (McLaughlin *et al.*, 1972).

Equation 9 can also be used to predict the membrane conductivity of gramicidin from the extraction equilibrium constants of gramicidin and, for example, nonactin and the conductivity of membranes containing nonactin. Assuming the u^* ratio for gramicidin to nonactin is 5 and using the data of Krasne *et al.* (1971) lead to the prediction that the conductivity of gramicidin-containing membranes should be well below the conductance of membranes in the absence of ion transporting substances which was 10^{-9} ohm^{-1} cm^{-2}. Gramicidin should not render membranes permeable to ions if it acts only as a diffusional carrier. However, the conductivity of membranes containing gramicidin is 10^{-3} ohm^{-1} cm^{-2} showing that gramicidin must act by a different mechanism from the diffusional carriers. These results are consistent with the postulate that gramicidin is a channel mediating antibiotic and not a diffusional carrier.

The ratio of occupied gramicidin pores to unoccupied pores has been calculated by Lauger for conducting lipid bilayers at maximum conductivity, where the $[M^+]$ was 10^{-3}–10^{-1}

$$K_0 = [\text{occupied pores}]/[M^+][\text{unoccupied pores}]$$

He obtained K_0 values of 3.3 for Na^+ and 4.3 for K^+ (Lauger, 1973). The corresponding value when using nonconducting organic solutions can be calculated using the data in Table II; $[M^+] = 0.911$, $C_{Pic}^{Tot^*} = 2.59 \times 10^{-6}$, and $C_G^* = 1.97 \times 10^{-4}$ gave $K_0 = 1.44 \times 10^{-2}$. In membranes, which are less polar than CH_2Cl_2 (Eisenman, *et al.*, 1968), one would expect an even smaller value of K_0. Thus, under conditions where gramicidin is conducting ions through membranes there are substantially more ions in the channel than when it is not conducting. Under nonconducting conditions the alkali metal ions are more stable in water solution than in the pore, while under conducting conditions Lauger's calculations show that the ions are more stable in the pore than in aqueous solution. This discrepancy may be due to the fact that the ratio of occupied to unoccupied pores is potential dependent as was suggested for the association constant of neutral carriers with ions (Hladky, 1972).

TABLE V: Summary of Ion Transport Experiments.

Picrate Side[b]		Organic Layer						
$[K^+]$ (M)	10^5 [Picrate] (M)	Component	Solvent	Concn $\times$ 10^4 (M)	Stirred	Time (hr)	% Transport	K_t[a]
0.1096	5.63	Gramicidin	CH_2Cl_2	2.17	Yes	95	38.9	7.86×10^8
8.96×10^{-5}	7.38	Gramicidin	CH_2Cl_2	2.51	No	97	<0.2	$>3.0 \times 10^5$
0.1040[c]	5.24	Gramicidin	CH_2Cl_2	2.51	No	98	70.9	1.00×10^8
8.96×10^{-5}	7.38	Gramicidin	$CHCl_3$	1.92	No	101	<0.2	$>3.0 \times 10^5$
8.96×10^{-5}	7.38	Crown	$CHCl_3$	1.92	No	95	14.2	4.7×10
8.96×10^{-5}	7.38	Crown	$CHCl_3$	1.92	Yes	119	55.1	0.99
0.1096	5.63	Gramicidin	$CHCl_3$	1.92	No	96	<0.2	$>2.3 \times 10^8$
8.96×10^{-5}	7.38	Gramicidin	$CHCl_3$	1.92	Yes	118	<0.2	$>3.0 \times 10^5$

[a] See text. [b] Anion was OH^- unless otherwise noted. [c] Anion was Cl^-.

It is also possible that gramicidin is in a different conformation in lipid bilayers since its CD and nmr spectra are quite solvent dependent. That this would be the source of enhanced ion affinity is inconsistent with our extraction measurements which show that as the solvent polarity decreases the extracting ability decreases.

Ion complexing ability is related to a number of factors including pore size, field strength of ligands, whether the agent is linear or cyclic, and whether the agent contains a carboxyl or other easily ionizable functionality. The poor ion complexing ability of gramicidin is probably related to some extent to all of these factors.

Gramicidin unlike the diffusional neutral carriers is linear and unlike the diffusional carboxylic acid carriers lacks a carboxyl group. A gramicidin-M^+ complex thus carries a net charge and the entropy loss which accompanies the wrapping of gramicidin around cation might significantly destabilize the complex. Apparently in the linear carboxylic acid diffusional carriers this entropy loss is compensated by the neutralization of the charge of the metal ion. Of course, if the most stable conformation of gramicidin is a helix, as suggested by Urry, then this entropy loss need not be considered (Urry, 1972).

An important factor which contributes to the poor ion binding ability of gramicidin is the looseness of fit of the ion in the helix pore. Corey-Pauling-Koltun (CPK) models of π (L,D) helices indicate that there are 15 carbonyl oxygen functionalities located near the center of the pore as well as many amide functionalities. The outer coat contains mainly hydrocarbon functionalities in marked similarity to diffusional carriers. Based on these considerations alone it is unclear why gramicidin does not bind ions as tightly as diffusional carriers. A characteristic of peptide-containing diffusional neutral carriers, crown ethers, and oxygen containing antibiotics is a rigid cyclic structure with a pore size accommodating one ion most favorably (*e.g.*, valinomycin, K^+; dicyclohexyl-18-crown-6, K^+; dibenzo-21-crown-7, K^+ and Cs^+; nonactin, K^+ and Rb^+). Membrane conductivity studies on nonactin (Eisenman *et al.*, 1968), for example, show similar selectivity (see discussion above relating ion complexing ability to membrane conductivity). The large binding constants of these neutral carriers for certain ions is in part related to the nearly "perfect fit" of cations in the central cavity as can be seen by comparing the binding constants in Table III with cavity sizes and ionic diameters in Table IV. In contrast, membrane conductivity experiments using gramicidin show that it transports Na^+ and Cs^+ with equal ease, and is relatively unselective.

These data can best be explained if gramicidin is considered either a "loose" helix with no fixed pore diameter or a helix with a large diameter, *e.g.* π^8(L,D), thus showing little ion selectivity and poor ion binding relative to neutral carriers which have fixed cavity sizes and show good ion binding to specific ions, and substantial ion selectivity.

Such an explanation is consistent with observations of Frensdorf that, for example, the K^+ ion binding ability of crown ethers measured in methanol, K, steadily increased as the pore size was increased from 14-crown-4, $K = 1.99 \times 10$, to 18-crown-6, $K = 1.26 \times 10^6$, and then decreased as the pore size increased further to a value of 3.09×10^3 for dibenzo-24-crown-8 (may be 2:1 complexing, see below). The comparable value for pentaglyme ($CH_3(OCH_2CH_2)_5OCH_3$), the linear version of 18-crown-6, was 1.48×10^2. Thus, a loose fitting cycle of ether functionalities binds ions only slightly more strongly than a linear polyether. The effect of loosening the fit of the ion in the cycle on the binding constant, K, is dramatically illustrated by comparing the structures and binding constants of the sodium and potassium complexes of dicyclohexyl-18-crown-6 (isomer B). The $Na^+\cdots O$ distances range from 2.7 to 2.9 Å in the Na^+-dicyclohexyl-18-crown-6 (isomer B) structure while the $K^+\cdots O$ distances range from 2.2 to 2.5 Å in many K^+-crown structures (Mercer and Truter, 1973a). The binding constant, K, of K^+-dicyclohexyl-18-crown-6 (isomer B) is 2.40×10^5, while the K of Na^+-dicyclohexyl-18-crown-6 (isomer B) is 4.79×10^3; thus loosening the cycle by 0.4 Å substantially decreases the binding constant, K. The cycle of dibenzo-24-crown-8 is so "loose" that it complexes two potassium ions in its central core which is oblong and measures roughly 7.5 × 3 Å (Mercer and Truter, 1973b).

Although our data do not allow differentiation between a gramicidin helix with variable diameter and a helix with a large diameter, the variable diameter helix is most consistent with membrane conductivity data. Membranes ranging from 27 to 47 Å showed identical NaCl conductance (Hladky and Haydon, 1972). A large diameter helix π^8(L,D) has a length of only 18–24 Å (Urry, 1971) for gramicidin dimers, and thus would probably be too short to conduct through long membranes. Studies of the ion selectivity of

thick membranes containing gramicidin might provide some experimental confirmation or rejection of these ideas since if the helix is elongated to transport ions 47 Å one would expect the helix to be a π^6(L,D), 26–30 Å long with a 4-Å pore size, or even approaching a π^4(L,D) helix, 35–39 Å, 1.4-Å pore diameter. If the helix is indeed elongated then one would expect that it might not transport large ions such as Cs^+ readily.

Ligand–ion interactions may also be important as suggested by a recent study of the ion selectivities of valinomycin and hexadecavalinomycin. Hexadecavalinomycin has a one-third larger cavity than valinomycin. Valinomycin and hexadecavalinomycin showed identical ion selectivities among group Ia cations representing a case where the ion–ligand interactions and not cavity sizes must be determining the selectivities (Eisenman and Krasne, 1974).

It is also possible that the electron pairs of the oxygen atoms in gramicidin are not pointing in a direction most favorable for ion complexation thus contributing to the low binding constant K. Studies of Mercer and Truter (1973a) of the geometry of complexation in the 18-crown-7 series as well as the electrostatic nature of the complexation suggest that this is probably not the major factor lowering the K^+–gramicidin binding constant.

From the data presented and discussed above one would expect that gramicidin would have very little, if any, ability to transport ions in U-tube experiments. In these studies a U-tube is filled with a chloroform or methylene chloride solution of gramicidin to above the bend, water is added to one side of the tube, and K^+Pic^- is added to the other side. After 4 days the amount of picrate transported to the water side is a measure of the ion transporting abilities of gramicidin or crown ether through these "long" membranes.

Table V summarizes the results of several U-tube experiments and the equilibrium constant is defined as shown

$$K_t = \frac{[K^+]_l[Pic]_l}{[K^+]_b[Pic]_b}$$

The concentrations on the side to which the picrate solution was originally added are in the numerator. The denominator expresses the concentration of the components on the side which originally contained only water. The value for K_t at equilibrium is theoretically 1 and was obtained in one experiment using crown dissolved in chloroform.

These U-tube experiments show, as predicted, that gramicidin transports ions through "long" membranes more slowly than crown ethers. In chloroform gramicidin does not transport a measurable amount of ions while crown ethers show substantial transport. In the more polar solvent methylene chloride, gramicidin transports ions, as would be expected since gramicidin does extract about 1% of the picrate ions into the organic layer under these conditions. The failure of gramicidin to transport ions through chloroform suggests that there should be membranes too thick for gramicidin to render them permeable to ions. This is consistent with the data of Hladky who found that 64-Å lipid bilayers containing gramicidin had no ion conductivity (Hladky and Haydon, 1972).

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Fractionation of Synaptic Plasma Membrane Glycoproteins by Lectin Affinity Chromatography†

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ABSTRACT: Synaptic plasma membranes were prepared from rat brain and extracted with 1% DOC–5 mM Tris-HCl buffer (pH 8.4), and the extract was chromatographed on an affinity column consisting of *Lens culinaris* phytohaemagglutinin attached to Sepharose 4B (LcH Sepharose). Most of the 7–8 glycoproteins present in the membrane extract were retained by LcH Sepharose and eluted with 2.5% α-methyl D-mannopyranoside. When a DOC extract of synaptic membranes labeled with [^{3}H]fucose was chromatographed on LcH Sepharose 45% of the radioactivity was retained; 25% of the radioactivity was retained when a similar extract was applied to an affinity column made with wheat germ agglutinin (WGA Sepharose). A membrane extract labeled with [^{3}H]fucose was chromatographed on LcH Sepharose and WGA Sepharose columns which had been connected in sequence. Under these conditions four fractions containing [^{3}H]fucose labeled glycoproteins were obtained. Sodium dodecyl sulfate gel electrophoresis indicated that each of the four fractions possessed a characteristic glycoprotein composition. The results are discussed in terms of the composition and possible functional significance of the membrane glycoproteins. The general applicability of the procedure for the purification of both membrane and soluble glycoproteins is suggested.

Glycoproteins are present as constituents of cell surface (plasma) membranes and have been implicated in a variety of surface related phenomena (see review by Hughes, 1973). The number, nature, and distribution of surface glycoproteins have been investigated by studying the reaction of lectins, proteins which bind in a highly specific manner to sugar residues (Sharon and Lis, 1972), with the surface membrane (Cuatrecasas, 1973; Henning and Uhlenbruck, 1973; Nicolson and Singer, 1971; Sela *et al.,* 1971; Fox *et al.,* 1971). Carbohydrates are present on the surface of neuronal cells (Rambourg and Leblond, 1967; Pfenninger, 1973) and receptors for the plant lectin concanavalin A have been demonstrated on the surface of isolated synaptosomes as well as in the synaptic cleft (Matus *et al.,* 1973; Bosmann, 1972). Isolated synaptic plasma membranes are enriched in protein bound glucosamine and sialic acid (Gombos *et al.,* 1971) and have been shown by gel electrophoresis to contain six to eight molecular weight classes of glycoproteins (Gurd *et al.,* 1974; Banker *et al.,* 1972).

Lectins have been used for the isolation of both soluble and membrane associated glycoproteins (Allen *et al.,* 1972; Lis and Sharon, 1973). As an initial step in a study of the functional and biosynthetic properties of synaptic plasma membrane glycoproteins we now describe their isolation and fractionation by affinity chromatography using the lectins isolated from *Lens culinaris* and wheat germ as the affinity ligands. A preliminary report of this work has been given (Gurd and Mahler, 1974).

Materials and Methods

Preparation of Synaptic Plasma Membranes and Administration of Isotope. Synaptic plasma membranes were prepared from the cortices of 30–35-day old Sprague Dawley rats as previously described (Gurd *et al.,* 1974). For experiments in which the distribution of [^{3}H]fucose was determined the isotope (250–500 μCi) was taken to dryness under N_2 at 4°, dissolved in 50 μl of 10 mM PO_4/0.9% NaCl (pH 7.6), and administered by intracerebral injection of 10 μl of this solution to each of five rats. After 16 hr the animals were killed, labeled cortices were mixed with an equal number of unlabeled cortices, and plasma membranes were prepared as above.

Isolation of Lectins and Preparation of Affinity Columns. Affinity columns were prepared using lectins isolated from the common lentil, *Lens culinaris* (LcH),[1] and from wheat germ (WGA). LcH was isolated essentially as described by Hayman and Crumpton (1972) using adsorption of the lectin on G-100 as the primary purification step. A Mn^{2+} requirement for LcH has been described (Paulová *et al.,* 1971) and it was necessary to include 5 mM $MnCl_2$ in all solutions used during the isolation procedure in order to minimize the loss of carbohydrate binding activity. WGA

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[1] Abbreviations used are: LcH, *Lens culinaris;* WGA, wheat germ; SDS, sodium dodecyl sulfate; DOC, sodium deoxycholate; GlcNAc, *N'*-acetylglucosamine; αMDMP, methyl α-D-mannopyranoside; PAS, periodate-Schiff procedure.