DEVELOPMENT OF K^+ - NA^+ DISCRIMINATION IN EXPERIMENTAL BIMOLECULAR LIPID MEMBRANES BY MACROCYCLIC ANTIBIOTICS

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Adsorption of macrocyclic antibiotics of the valinomycin, actin and enniatin groups to experimental bimolecular lipid membranes increases the membrane conductance up to 10^8 times. The single ion conductances differ by as much as 300 times increasing in the order Li < Na < Cs < K < Rb, and resting potentials up to 150 mV result when 0.1 M solutions of NaCl and KCl are placed on opposite sides of the membrane. The observations suggest that appropriate ring diameters permit hydrogen bonding carbonyl oxygens in the ring to substitute for water molecules of the hydration shells of the cations. In general, cations with higher field strengths and solvation energies show larger activation energies and poorer conductances. The ring compounds could act as both pores and carriers depending on conditions.

Methods and Results

Figure 1a shows that the conductance in lipid bilayers formed from beef brain lipid by methods given elsewhere (Mueller, Rudin, Tien and Wescott, 1963) increases in a few minutes from an intrinsic value of 10^{-8} mhos cm^{-2} to 10^{-3} mhos cm^{-2} upon adding 10^{-7} gm/cc valinomycin, a cyclododecadepsipeptide, to one or both sides of the membrane in the presence of 0.1 M KCl on both sides; 10^{-9} to 10^{-10} gm/cc has a detectable effect. The conductance usually rises linearly for 3 - 10 minutes and may go through a maximum before reaching a stable value.

The cyclohexadepsipeptides, enniatins A and B, the tetralactones, monactin and dinactin and gramicidin A, B and C are also effective but the enniatins require a higher concentration (100 X). All act on membranes made from a large variety of lipids including mixed beef brain or heart lipids, purified lecithin or sphingomyelin. They are also active (Harris, 1966) in bilayers composed of oxidized cholesterol derivatives (Tien, Carbone and Dawidowicz, 1966). The rate and degree of conductance change depends on the lipid type

and additives used in making the membrane solution. Tocopherol increases membrane responsiveness while cholesterol often depresses it although the enniatins seem to be more effective in pure oxidized cholesterol membranes (Harris, 1966). The membrane capacity remains constant (0.7 $\mu F\ cm^{-2}$) during the conductance changes (Brattain, 1966).

If identical monovalent salts at two different concentrations are placed on opposite sides of the membrane, the antibiotics induce resting membrane potentials which are negative on the side of the higher concentration by values of 54 - 58 mV per decade. Consequently, the selectivity, i.e. the permeability ratio for cation:anion is large and may approach infinity.

If the membrane separates 0.05 M NaCl from 0.05 M KCl, potentials up to 150 mV negative on the side of the K^+ result as shown in Table 1. Using valinomycin as representative a Nernst plot is given for these conditions in Figure 1b. It indicates that the permeability is about 400 times greater for K^+ than for Na^+.

Table 1. Ionic Selectivity Data

		Li	Na	Cs	K	Rb
Biionic potential E_{AB} in mV	Val	0	8	135	151	172
	Enn		0	21	90	
	Din	0	5	35	85	110
	Gram	0	15	55	60	65
Selectivity coefficient $K_{AB}=P_A/P_B$	Val	1	1.4	210	395	920
	Enn		1	2.3	37	
	Din	1	1.2	4.1	30	82
	Gram	1	1.8	8.8	11	13
Single ion cond. ratios g_A/g_B (35°)	Val	1	1.2	50	> 200	> 300
	Gram	1			20	
Biionic cond. ratio g_{A+B}/g_A	Val	2.0	1.8	0.63	0.5	0.44
	Gram				0.5	
Single ion act. energies (Kcal/mole)	Val		61.0		30	
	Din		35		20	
	Gram				28	

Val = 3 unit valinomycin, 10^{-6} gm/cc; Din = Dinactin, 10^{-6} gm/cc; Enn = Enniatin B, 10^{-5} gm/cc; Gram = Gramicidin A, 10^{-6} gm/cc. Observed biionic potentials (E_{AB}) at 0.05 M. The selectivity coefficients (K_{AB}) are derived from E_{AB} by: $K_{AB}=P_A/P_B=[A_o]/[B_i]\exp E_{AB}F/RT$, where P_A, P_B are the permeability constants for the ions A and B; $[A_o]$, $[B_i]$ are the ion activities in the outside and inside compartments and in all cases in the Table B_i is Li^+; F/RT has the usual meaning. The observed single conductances, g_A, for ion A inside and outside at 0.05M are given as the ratio with respect to g_{Li^+}. The activation energies were calculated from the temp. coefficients of g_A which are constant between 22 and 45° C. The biionic conductance ratios are defined as the single ion conductances of the equimolar mixture of that ion with Li^+, i.e. g_{A+B}/g_A where [A]= [B]=0.05 M and B= Li^+. Under single ion conditions the current-voltage curve is ohmic; under biionic conditions it shows typical Goldman rectification. The values are representatives from 5 - 10 experiments. Lipid solution composition: Sphingomyelin 2.5 % (Applied Science or Pierce) in α-tocopherol :$CHCl_3$:CH_3OH, 5:3:2. The sources of the antibiotics were: Natural valinomycin (L. C. Vining through J. C. McDonald), synthetic valinomycins (M. M. Shemyakin through B. C. Pressman), enniatins (Pl. A. Plattner), actins

(Squibb and B. C. Pressman) and gramicidins (L. N. Craig). Divalent ions show little effect with these compounds.

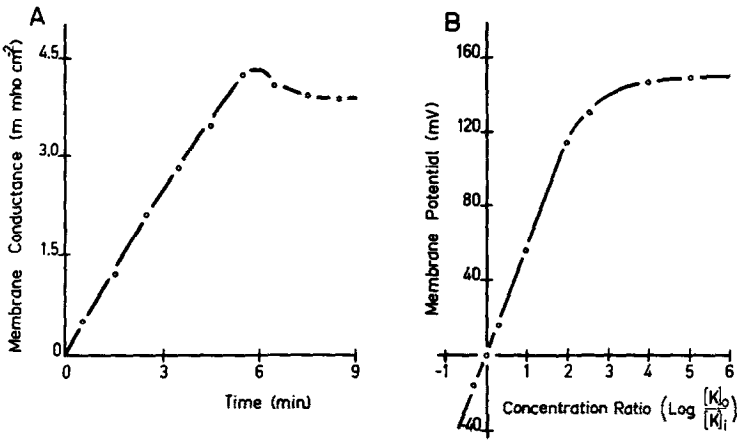

Figure 1.

(a) Time conductance curve developed by 10^{-6} gm/cc valinomycin added at zero time to a bilayer of crude beef heart lipids with 0.1 M KCl on both sides of the membrane. (b) K^+/Na^+ specificity induced by valinomycin. The membrane made from beef heart was formed in 5 mM/l histidine Cl buffer. KCl was added inside and NaCl outside to 0.1 M/l each, i.e. $(K_i) = (Na_o) = 0.1$ M/l. Synthetic valinomycin (10^{-5} gm/cc in ethanol) was added to the inside to give 10^{-7} gm/cc. The membrane resistance fell from 10^8 to 500 Ωcm^2 and a resting potential of 150 mV appeared within 30 seconds. KCl was then added to the outside to give the indicated ratios of K_o/K_i. The ethanol solvent is without effect. Conductances and potentials were measured by circuits given elsewhere (Mueller et al, 1963).

For dinactin the conductance effects and specific ion potentials are similar in colored membranes~5000 Å thick in which bilayer formation has been suppressed by adding tetradecane to the lipids. Valinomycin produces some conductance increase in these thick membranes but the large bi ionic potentials appear only in bilayers where the conductance effects are also 10 fold larger. Gramicidin acts only in bilayers.

The effectiveness of natural and synthetic valinomycins of different ring size, amino acid composition and DL configuration in lowering the bilayer resistance correlates with their effect on mitochondrial ion transport (Pressman, 1965) and antibiotic activity (Shemyakin et al, 1965). Either increasing the ring diameter from 3 to 4 units or decreasing it to 2 units depresses the selectivity and conductance effects. Increasing the number of hydrocarbons and the lipophilic balance by substituting leucine for alanine increases the effectiveness. Cyclic compounds with polar groups on the outer ring diameter such as filipin, colimycin or amphotericin were without effect. The $K^+ - Na^+$ selective properties of all the active ring compounds are similar

in mitochondria (Pressman, 1965; Harris, Cockrell and Pressman, 1966) and in the experimental bilayers.

Discussion

As ion selective electrodes, the macrocyclic-bilayer system equals the selectivity shown by biological membranes and exceeds by one to two orders of magnitude the K^+ selectivity of other known cation sensitive systems, e.g. aluminosilicate glass electrodes (Eisenman, Rudin and Casby, 1957).

Because the ionic selectivity does not vary with the lipid type the effect is apparently an intrinsic property of the ring and not the result of induced rearrangements of polar groups of the lipids. The rings are devoid of charged groups and act in noncharged cholesterol bilayers. Consequently, theories invoking fixed charges or charged carriers are not likely to be applicable. Although some ring types can apparently act as noncharged carriers in thick lipid membranes, the tendency of these compounds to aggregate in solution and the fact that gramicidin and valinomycin produce their ion selectivity only in bilayers raises the possibility that high levels of aggregation and ion transfer from ring to ring could occur in bilayers to produce true pores, the carrier and pore concepts being taken as limiting cases determined by the degree of aggregation .

In either case the molecular models in Figure 2 show that outwardly directed hydrocarbon groups could interact with bilayer lipids by van der Waals forces while centrally directed proton acceptor carbonyl oxygens of valinomycin and nonactin just contact protons of the tetrahedrally coordinated first hydration shell around the cations. Carbonyl groups, e.g. in peptide bonds, have heats of hydration about 1.5 Kcal/mole greater than the internal energy of the water-water bond and therefore would compete favorably in solvating the cations. Anions would be excluded because of the inverted polarity of their hydration shell.

Larger (4 unit) or smaller (2 unit) valinomycin rings show little activity. In the first case the molecule becomes loose and unstable as a ring. In the second case none of the alkali metal ions, either naked or with their first hydration shells fit properly into the ring except for naked Cs^+. However, as the ring is reduced further to the size of the enniatins the rings become active again as they become able to accomodate the fully dehydrated cations within six closely coordinated carbonyl oxygens. Cs^+ generally occupies an exceptional position and shows some steric hindrance in the molecular models whether partially or fully dehydrated and this effect is more pronounced with enniatin and dinactin (Table 1). Although the gramicidins are linear molecules, infrared masking of the terminal hydroxyl functions (Sarges and Witkop) suggests that they may form rings.

Evidently, steric factors can account for the above effects. But ring flexibility over smal-

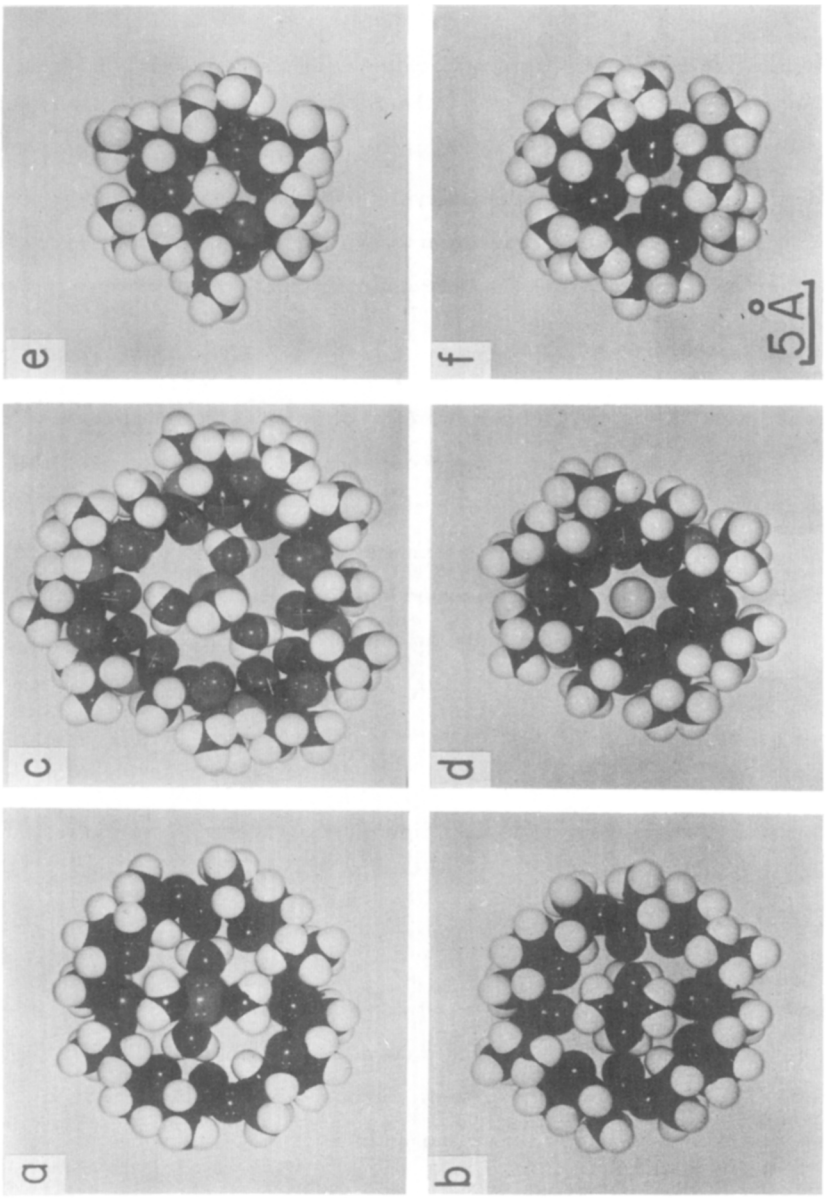

Figure 2.

Steric relations between macrocyclic antibiotics and ions. (a, b) Nonactin, $[O-\underset{\underset{O}{\|}}{C}-\underset{Me}{\underset{|}{C}}-\underset{\underset{O}{\|}}{C}-\underset{Me}{\underset{|}{C}}]_4$. Slight rotation of the carbonyl oxygens allows them to contact hydrogens of the first hydration shell tetrahedrally coordinated around Cs^+ (a) as well as Li^+. (c) Valinomycin, [D-Val L-Lact L-Val D-Oxisoval]$_3$ contains 12 carbonyls. The ring aperture is very similar to that of dinactin and also sufficiently flexible to fit all the hydrated cations (Cs^+ shown). The larger number of oxygens in the ring could account for larger activation energies. (d) Valinomycin (2 units) is too small to accommodate the hydration shell and too large to coordinate around the ion (K^+ shown). (e, f) Enniatin, [[L-Me·Val D-Oxisoval]$_3$] shows 6 fold coordination with Cs^+ (e) and Li^+ (f).

ler dimensions permits all the cations to be accommodated almost equally well by a ring of appropriate diameter (Figure 2). Consequently, the differential cationic selectivities must be sought in additional considerations. The low values of the single ion conductances for Li^+ and Na^+ together with the fact that these ions actually decrease the biionic conductances of K^+ and Rb^+ about 2 fold at equimolar concentrations indicate that the ions with poorer conductances cause channel or carrier blockage and that the equilibrium ion distribution ratios for all the ions are about one. Therefore the equilibrium distribution free energies are tentatively considered relatively unimportant in controlling the selectivities although direct experimental evidence has not yet been obtained. In contrast, the observed Q_{10} and therefore the activation energies and single ion conductances show large differences between the ions which correlate qualitatively with the biionic potentials although there are evidently other factors that prevent a simple relation. The specific chemical features of the rings imply that the activation energies are caused by ions entering and leaving the rings and not by the motion of the rings in the lipids. Therefore, it seems that the geometry and adaptive properties of the rings allow them to provide primary and secondary solvation shells the energies of which increase directly with the field strengths of the ions, causing the differential activation energies and conductances, as occurs in free solution. But the ring compounds amplify all these qualities over the values found in water because of the rigidity of the solvation shell which they provide. Hydrogen bonding pores of this general type have been proposed by Danielli and Davson (1934) for sugar transport and by Mullins (1959) for ions, although the proposed ionic selectivity mechanisms differed in detail.

It has been suggested that macrocyclic antibiotics alter the ionic distribution and uncouple the oxidative phosphorylation of mitochondria by increasing their membrane ion permeability (Chappell and Crofts, 1965; Pressman, 1965). Although this is supported by the similarity of their action in experimental bilayers it does not exclude the possibility that they also modify special receptor mechanisms such as ion pumps (Pressman, 1965). Both effects could occur (Pressman, 1965), their ratio being determined by the differential adsorption energies of the compounds for pure lipid regions vs regions already modified by other adsorbates in normal cell membranes.

The conductance of a proteinaceous compound from bacteria which induces electrical excitability in bilayers (EIM) and provides a basis for constructing action potentials (Mueller and Rudin, 1966) could also result from a structure containing hydrogen bonds. But the variation of the conductance with voltage may require the additional presence of charges. In fact, certain charged amphophilic compounds such as dipicrylamine and tetra-phenylboron have been found to generate voltage dependent permeability changes in lipid bilayers (Mueller and Rudin, 1967).

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