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STRUCTURAL EFFECTS IN THE ACTION OF ANTIBIOTICS ON THE ION PERMEABILITY OF LIPID BILAYERS

I. TYROCIDINE B

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SUMMARY

1. As a model for the interaction of proteins and lipids in membranes, the ion-carrying properties of the cationic decapeptide tyrocidine B in phospholipid bilayers were studied.

2. They differ in three principal ways from that of the neutral carriers valinomycin and monactin.

3. The presence of cholesterol (1:1 molar ratio) makes the kinetics bimolecular.

4. The presence of amines in the aqueous phase increases the Na^+ conductance relative to K^+, H^+.

5. H^+ conductance is not rate limited by surface reaction, and is increased by more hydrophobic amines.

6. The results in general indicate that there is interference between ions on the carrier structure which may be allosteric in nature.

7. If the affinity for the single ions is very different, this opens up the possibility of control of ionic conductance by the presence of a smaller quantity of another ion; and further, by a field dissociation (Wien) effect, may explain the switching phenomenon obtained with other more highly associative antibiotics.

INTRODUCTION

The action of antibiotics on lipid bilayers have provided useful models of undoubtedly more complicated physiological membrane permeation mechanisms. The latter, as presently understood, have been reviewed by EISENMAN[1] and PRESSMAN[2]. Among the neutral carriers only valinomycin[3] and the macrolide actins[4] have been studied sufficiently systematically to correlate their action with structure. IVANOV *et al.*[5] and OHNISHI AND URRY[6] using NMR have given a structure which accounts for K^+ specificity of valinomycin on the basis of hydrophilic cavity size, while for the actins the exceptional specificity for NH_4^+ found by SZABO *et al.*[4] could be explained by the approximate cubic symmetry of the model of KILBOURN *et al.*[7] as opposed to the 3-fold (S_3) symmetry of valinomycin. This is the first indication that ion structure as well as size and hydration play a role in ion specificity.

Another source of specificity can be the lipid composition of the bilayer. Thus for the pore-forming polyene antibiotics, cholesterol appears to be necessary[8,9]; however, its action on the macrolide actins is a large reduction in mobility without change in specificity[4]. So, except for some semi-quantitative results with liposomes[10] showing effects of sterols, the biologically important question, what kinds of lipid-ion carrier specificities exist, remains open, even for the simplest systems.

As a first approach to this problem the action of the cyclic decapeptide tyrocidine B known from a report of BEAN[11] to give ion-specific conductance, was studied in relation to the above parameters. It contains[12] an ornithine (α,δ-diaminovaleric acid) residue with a protonated amino group ($pK = 8.65$) and thus has H^+-carrying capacity which among other things supplies a useful reference from which to estimate association with other ions.

METHODS

These were similar in most respects to those described by SZABO *et al.*[4]. The 2-compartment (each 10 ml) chamber was milled out of 1.5-inch teflon cube. The separating septum was countersunk one side to a 1.4-mm diameter aperture and flat the other allowing the use of a flattened teflon tube, containing the lipid sample, which could then be drawn by a rack and pinion type of manipulator, across the aperture to spread a film. For flexibility in choice of buffer anions, agar-saturated KCl–AgCl–Ag electrodes were used with no excess fluid and small junction area (to avoid leakage) giving a total resistance of $2.5 \cdot 10^4\ \Omega$ in 1 mM KCl. Temperature was maintained at 35° by means of an infrared lamp. The whole apparatus was mounted in clean-air canopy (Dexon Corp., Minneapolis).

Phospholipid, consisting mainly of phosphatidyl choline, but containing traces of ethanolamine and inositol phosphatides, was prepared from soy bean extract (Asolectin, Associated Concentrates) by extraction with chloroform–methanol (5:7, by vol.) and precipitation with excess (10:1, v/v) acetone. All solvents were glass distilled and preparations done under a clean-air canopy. After draining off acetone the residue was dried under high vacuum and then without releasing the vacuum enough *n*-decane was admitted to give a final concentration of 100 mg/ml. This stock solution, kept at –10°, appeared to be stable against oxidation for about a month. For phospholipid it was diluted to 30 mg/ml and for phospholipid–cholesterol or phospholipid–cholestenone to 20 mg/ml *plus* 10 mg cholesterol (standard for chromatography, Sigma Chemical Co.) or cholest-4-ene-3-one (Eastman).

Water, deionised to 5 $M\Omega \cdot cm$ to remove NH_3, was distilled from a small still straight into the chamber *in situ*. Salts were added as concentrated solutions using a 10-μl pneumatic micropipette (Eppendorf). Tyrocidine (Rapicidin, Lot No. 106, Wallerstein Co.) was dissolved to 10 mM in methanol and diluted to $1 \cdot 10^{-3}$–$1 \cdot 10^{-6}$ M in water immediately before each addition, in view of its known[13] tendency to form large micellar complexes in aqueous solution. Further, in view of the possibility that contamination[13] with gramicidin S might obscure the results, it was established, using a sample of the latter purified by countercurrent distribution, that the threshold concentration for this antibiotic to produce a measurable conductance increment was at least two orders of magnitude higher.

Stirring was accomplished by miniature magnetic spin-bars. Before use,

chamber and glassware were subjected to a cleaning sequence of chromic acid, alkaline detergent (Hemosol), deionised water (large volume for 24 h), acetone, chloroform. With these precautions bilayers having a surface resistance of $3 \cdot 10^8$–$5 \cdot 10^8$ $\Omega \cdot cm^2$ were obtained and this was adopted as criterion of freedom from contamination. In the following the front chamber, containing the inspection window and grounded, will be called 'outside', the other 'inside'; and the sign of potential correspondingly. A glass electrode in the outside monitored the pH; unless otherwise stated this was 7.2, the antibiotic concentration $2 \cdot 10^{-7}$ M, and lipid, phospholipid–cholesterol.

Potential and resistance were measured with a Keithley 610B electrometer, using the constant-current generator for the latter. The measured open circuit resistance of the electrometer *plus* associated connections was $1 \cdot 10^{12}$ Ω. A fiber optic light source, held by a rack and pinion manipulator was adjusted to give specular reflection from the bilayer which was observed with a binocular microscope (Bausch and Lomb). A micrometer eyepiece was used to measure the effective diameter of 'black' lipid.

RESULTS

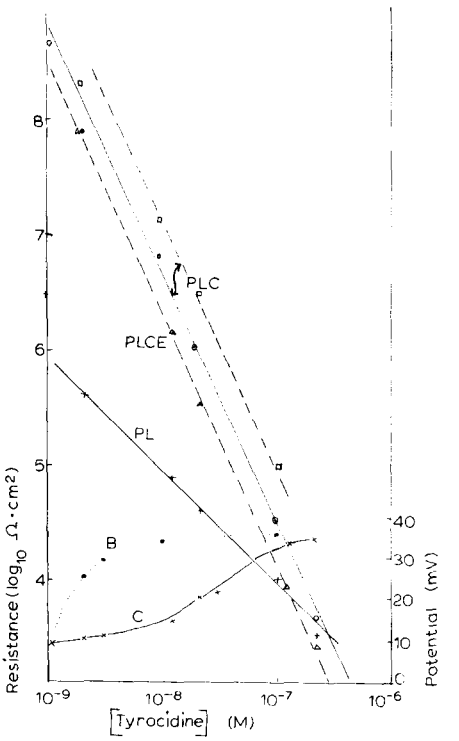
Fig. 1 shows dependence of resistance on tyrocidine B concentration at low (1.45 mM) salt concentration. For phospholipid the slope corresponds to a specific conductance (=specific surface conductance per equiv cation per molarity antibiotic), referred to K^+, $\sigma_{KTyr} = 4.6 \cdot 10^6$ $\Omega^{-1} \cdot mole^{-2}$. This lies between $\sigma_{KNon} = 8 \cdot 10^5$ $\Omega^{-1} \cdot mole^{-2}$ for nonactin[3] and $\sigma_{KVal} = 11 \cdot 10^6$ $\Omega^{-1} \cdot mole^{-2}$ for valinomycin[3] (estimated extrapolation to low salt concentration). For phospholipid–cholesterol and phospholipid–cholestenone, however, the reaction is quite closely bimolecular.

In view of this, a difference in ion specificities was looked for, again with Tris as the common (to both sides) cation. Fig. 2 shows that although an inversion of expected sequence $NH_4^+ > K^+ > Na^+$ of permeabilities was observed this was not altered by phospholipid–cholesterol, rather the transport ratio of each ion was lowered with respect to Tris. This lead to the suspicion that the latter was responsible for the inversion itself; in fact this can be concluded by comparing the lower Na^+ conductance relative to Tris of Fig. 1 to the potential developed by Na^+ in the presence of Tris of Fig. 2. Measurement of bionic potentials in 1 mM phosphate buffer showed $K^+ \sim NH_4^+ > Na^+$ with $V_{KNa} = +35$ mV, however, the other pair of potentials, $V_{KNH_4} = +6$ mV and $V_{NH_4Na} = +19$ mV, were not additive in a sense indicating that NH_4^+ causes selective increase in the Na^+ conductance.

The use of buffers was based on the assumption H^+ conductance would be high. Fig. 3, Curve B, shows that this is not the case, in the fact the bionic transport ratio τ_{HK} $(= e^{eV_{HK}/kT}) = 20$, the same value found by LEV AND BUZHINSKY[3] for valinomycin. This means that it is possible to examine pH dependence, in the range 4.5–7.2 of ion conductances unmasked by H^+ conductance. Acetic acid was used to change the pH because of its buffering capacity, however, the results were not essentially different using HCl, so that the H^+-carrying capacity of the former[14] was not in question.

Fig. 3, Curve A, shows that K^+ conductance is inversely proportional to the concentration of H^+ indicating that one-half of the dimer carrier is contributed by

the uncharged species. This fact accounts for the rapid approach to the Nernst slope (58 mV/pH unit) at pH 4.5 and allows one to measure τ_{HK} and τ_{HNa} in the presence of amines by working in this region. Curves C and D, Fig. 3, show that assuming the usual Goldman type[16] of relation between conductance and potential, Na^+ conductance is increased 2.5 times (corresponding to a pH shift of 0.4 pH unit in the 'Nernst' region) in the presence of NH_4^+, while the corresponding Curve B shows effectively no shift. This may be interpreted to mean either there is no 'amine effect' for K^+, H^+ or that they are the same. The latter is ruled out by the apparent increase in Na^+ conductance by this method being not less (actually it is greater) than that given by the bionic potential. However, this is not true for other amines.



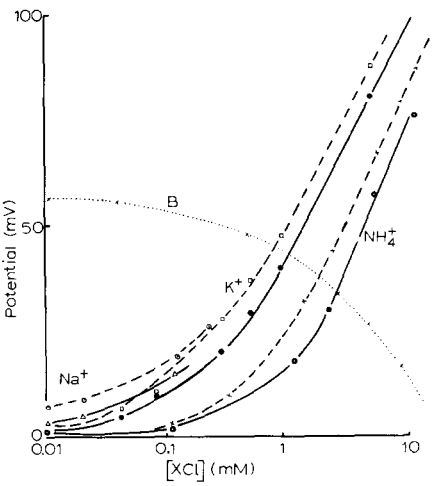


Fig. 1. Bilayer surface resistance in the presence of 1.45 mM Tris buffer for designated lipids, and varying tyrocidine B concentration. Curves B and C record V_{KNa} as function of tyrocidine B concentration, inside and outside, respectively. ×—×, phospholipid, △——△, phospholipid–cholstenone, ○—○, phospholipid–chlolesterol; □----□, phospholipide–cholesterol in sodium phosphate buffer.

Fig. 2. Potential developed by designated cation X = Na^+, K^+, NH_4^+, outside; 1.45 mM Tris both sides. Dashed lines, phospholipid; solid lines, phospholipid–cholesterol. Curve B, 1.10^{-7} M tyrocidine B outside and XCl = KCl, inside. pH 5.5.

Curves E and F show that the effect of trimethylammonium is now also on the H^+ conductance leaving the relation between the Na^+ and K^+ conductances the same as in the presence of NH_4^+.

That the tyrocidine B-mediated H^+ conductance is not rate limited at the bilayer surface is shown, Fig. 2B, by the potential, generated by tyrocidine B alone

outside in distilled water (pH 5.5), representing the effective concentration ratio across the membrane. The depence of potential on KCl added inside is consistent with $\tau_{HK} \sim 30$. All the other situations above were indifferent to the presence of tyrocidine B on one, rather than both sides. However, this was fortuitous in view of the result of Figs. 1B and 1C for V_{KNa} at other concentrations which shows a marked dependence on which side the tyrocidine B is present.

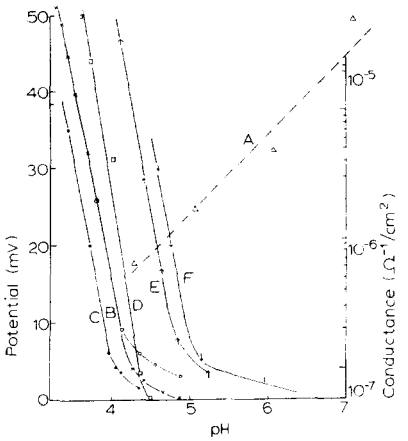


Fig. 3. A, initially 1 mM potassium phosphate buffer, pH lowered equally at both sides by addition of acetic acid. Conductance measured on righthand scale. Curves B–F, following ion pairs (outside, inside, respectively). B (x), K^+–K^+; B (o), NH_4^+–K^+; C, NH_4^+–Na^+; E, $(CH_3)_3NH^+$–Na^+; F, $(CH_3)_3NH^+$–K^+; each initially as phosphates; pH lowered by acetic acid at outside only. Potential measured, lefthand scale.

DISCUSSION

That ion carriers which are (as for tyrocidine B) weakly selective may be modified by cofactors suggests this mechanism may be present in the action of biogenic amines as transmitter substances and possible has a role in active transport, of the type where one has Na^+/K^+ flux coupling[15].

The effect of lipid composition in Fig. 1 can be explained by the presence of two surface reactions in sequence. The first, adsorption, is monomolecular and normally (*i.e.* for phospholipid) rate limiting. The second, dimerisation depending on the surface mobility of carrier, is slowed up by cholesterol and becomes rate limiting at concentrations $> 3 \cdot 10^{-7}$ M antibiotic. The result of Fig. 2B appears to show that in this case diffusion across the bilayer is actually rate limiting, while the result of Fig. 1, Curves B and C, which shows the effect of tyrocidine B on one side only or the other, is the reverse of what would be expected on a simple carrier hypothesis[16] and seems to indicate that the ion predominantly transported requires the presence of another in the 'carrier'.

Thus, the interference of ions, which may be regarded as a kind of allosteric action, is not confined to one species being an NH_4^+. By modification of the Wien effect[17], *i.e.* field-dependent ion dissociation, this could give rise to the 'bistable' behavior that has been observed for the larger cyclic nonadecapeptide alamethicin[18]

and the cyclic hexadepsipeptide monamycin[19], where the order of the reaction is considerably higher, *i.e.* about 6 in both cases.

In general the results show a more complicated behavior than neutral carriers, although there is no conclusive evidence here of the pore or channel formation that is present with gramicidin A (unrelated to gramicidin S) which is also bimolecular in its action[20]. However it should be remembered that carrier and pore mechanisms are but limiting cases of a general mobile structure model and, therefore, such interpretations are presently rather tentative. A comparative study of cyclic peptides is indicated before general structural principles can be formulated and these will be the subject of further communications.

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REFERENCES

1 G. EISENMAN, *Federation Proc.*, 27 (1968) 1249.
2 B. C. PRESSMAN, *Federation Proc.*, 27 (1968) 1283.
3 A. A. LEV AND P. E. BUZHINSKY, *Tsitologiya*, 9 (1967) 102.
4 G. SZABO, S. CIANI AND G. EISENMAN, *J. Membrane Biol.*, 1 (1969) 346.
5 V. T. IVANOV, I. A. LAINE, N. D. ABDULAEV, L. B. SANYAVINI, E. M. POPOV, YU. A. OVCHINNIKOV AND M. M. SHEMYAKIN, *Biochem. Biophys. Res. Commun.*, 34 (1969) 803.
6 M. OHNISHI AND D. W. URRY, *Biochem. Biophys. Res. Commun.*, 36 (1969) 164.
7 B. T. KILBOURN, J. D. DUNITZ, L. A. R. PIODA AND W. SIMON, *J. Mol. Biol.*, 30 (1967) 559.
8 A. FINKELSTEIN AND A. CASS, *J. Gen. Physiol.*, 52 (1968) 145.
9 T. E. ANDREOLI AND M. MONAHAN, *J. Gen. Physiol.*, 52 (1968) 300.
10 A. D. BANGHAM, M. M. STANDISH AND G. WEISMANN, *J. Mol. Biol.*, 13 (1966) 253.
11 R. C. BEAN, *Philco-Ford Corp. Rept.* No. U-4184 (1967).
12 D. GOTTLIEB AND P. D. SHAW, *Antibiotics*, Vol. 1, Springer, New York, 1967, p. 636.
13 R. C. WILLIAMS, JR., *The Non-covalent Association of Tyrocidine B*, Ph. D. Thesis, Rockefeller University, 1967.
14 P. C. CROGHAM, G. J. A. LEA AND J. LELIEVRE, *J. Physiol.*, 200 (1969) 114P.
15 P. C. CALDWELL AND R. D. KEYNES, *J. Physiol.*, 155 (1960) 177.
16 S. CIANI, G. EISENMAN AND G. SZABO, *J. Membrane Biol.*, 1 (1969) 1.
17 L. BASS AND W. J. MOORE, *Structural Chemistry and Molecular Biology*, Freeman, San Francisco, 1968, p. 356.
18 P. MUELLER AND D. O. RUDIN, *Nature*, 217 (1968) 713.
19 M. C. GOODALL, *Nature*, in the press.
20 D. A. HAYDON, *3rd Intern. Biophys. Congr., Cambridge, Mass., 1969*, in the press.