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

II. KINETICS OF TYROCIDINE B

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SUMMARY

1. The kinetics of tyrocidine B action on phospholipid and phospholipid–cholesterol bilayer shows autocatalytic behavior described by an hyperbolic tangent function $\frac{1}{2}$ [1 + Tanh k_1^+ $(t—t_0)$] characterized by two constants: a rate k_1^+ and a latency parameter $\mu = \exp[-k_1^+ t_0]$.

2. These constants are studied as functions of transmembrane potential and concentration differences, as well as the usual kinetic parameters.

3. The results are summarized in a reaction scheme for which a minimal interpretation is that tyrocidine acts through bimolecular transmembrane structures, the formation of which is autocatalytic and rate limiting.

4. Prior to this a fast adsorption process is required which has a low ($< 1 \cdot 10^{-9}$ M) half-saturation constant. If it is assumed that the latter is ion dependent, being much smaller for K^+ than for Na^+, then anomalous results found previously for Na^+–K^+ bionic potential as function of antibiotic concentration are explained.

5. There is also a fast bimolecular reaction, which may take place in the aqueous phase. Conductance development requires that its product reacts with the transmembrane structures above, to give a "carrier complex".

6. These results are markedly dependent on the polarity of lipid used, indicating that both mechanisms under (3) and (5) above are influenced by the charge profile of the bilayer. The latter can be changed by adsorption of Ca^{2+} on the one hand or acidic glycolipid (ganglioside) on the other, added to the aqueous phase.

7. The dimers, referred to under (3) and (5) above, may be interpreted as nonpolar and polar associations respectively.

8. In all the above respects tyrocidine occupies a position intermediate between simple carriers and highly associative pore forming antibiotics.

INTRODUCTION

Equilibrium conductances and diffusion potentials obtained with this decapeptide were reported previously[1]. They showed significantly different behavior from

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that of neutral carriers[2]. Here the corresponding kinetics are studied to provide further clues as to mechanism of action. The situation is especially favorable for this since the kinetics are relatively slow and can be followed over a large dynamic range (at least 1–10000); furthermore, in addition to the usual kinetic parameters of concentration and temperature, one has both transmembrane potential and concentration differences. As a test of the resolution obtainable one can compare tyrocidine B with the structurally related gramicidin S; this will be done in a following paper.

METHODS

These were the same as in ref. 1 with the following modifications. Disposable liquid-junction electrodes were used consisting of lengths (3 cm) of 1.4-mm diameter melting point tube partially closed over at the open end by flame polishing and filled with saturated KCl–agar (3 %) pushed into rubber grommets on standard KCl–AgCl–Ag reference electrodes. Current was measured by a battery operated solid state meter (Keithley 602) inserted between the front chamber and ground, the output was recorded on a strip chart recorder (Mosely 680M) for time record, or X–Y recorder (Moseley 135CM) for current–voltage plots. Voltage was measured by electrometer (Keithley 610B) and supplied from a rechargeable cadmium cell or, for *I–V* plots, a variable-phase oscillator (Hewlett Packard 203A). A switching arrangement permitted the substitution of decade resistances up to $1 \cdot 10^9\ \Omega$ for calibration.

Egg lecithin, colorless and chromatographically pure, in decane (Applied Science Labs, State College, Pa.) was found to be very susceptible to oxidation, as evidenced by geling at low temperature, and was, therefore, kept under argon. The more stable phosphatidyl choline from soya bean, described in ref. 1, which apparently contains some natural antioxidant, was also found to be improved by passing through a short column of activated coconut charcoal (Fisher 6-585). Bacterial phosphatidyl ethanolamine containing cyclopropane bonds[3] in place of unsaturation was from *Escherichia coli*. In each case solutions of 30 mg/ml were diluted with octane until the rate of thinning gave black membranes in 2–4 min. Brain ganglioside (Sigma, Type III) was used as purchased. Unless otherwise stated, the aqueous phase was 1 mM potassium phosphate at pH 7.2 and 36.5° and the tyrocidine added both sides, as 10 μl of methanol solution. In the only one-sided experiment (Fig. 9) this fact is noted explicitly.

RESULTS

Equilibrium conductances were remeasured in 1 mM potassium phosphate (rather than Tris) buffer using constant voltage (20 mV) allowing sufficient time (about 15 min) to reach equilibrium. The results (Fig. 1) confirmed the statements of ref. 1, with exception of phospholipid without cholesterol which now gave a log–log slope of 2; this was not due to the different electrolyte (potassium phosphate in place of Tris–HCl) but to the following artifacts in the previous measurements: (i) lipid contamination at low concentration of antibiotic; (ii) electrode resistance at high conductance; (iii) failure to take into account kinetics of conductance development and its dependence on applied voltage. Each of these sources of error has been progressively eliminated; in particular the continuous voltage recording method used here, as opposed to the previous constant current method, allows one to check these most easily.

The considerably (350×) lower conductances with bacterial phosphatidyl ethanolamine is noteworthy. This lipid showed in the untreated bilayer a marked anion selectivity (P_{Cl}/P_K approx. 100).

The approach to equilibrium under the same conditions is shown in Fig. 2. The symmetry about the midpoint suggests a hyperbolic tangent function, $g/g_{max} =$

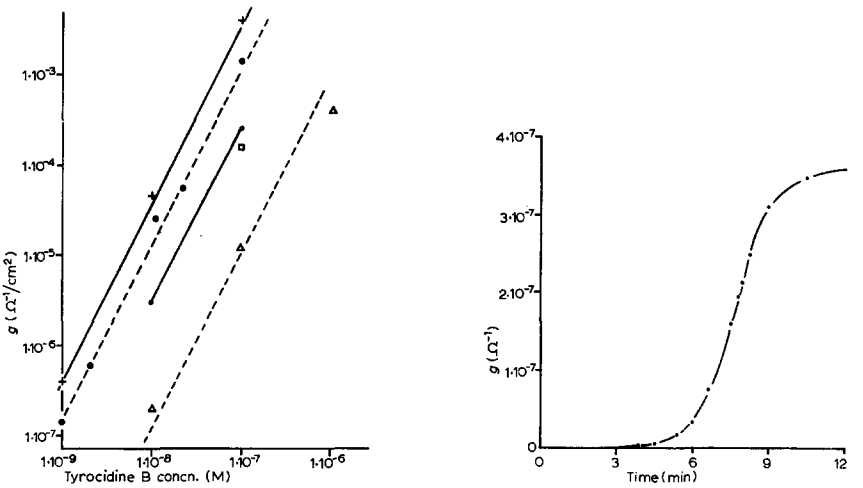

Fig. 1. Equilibrium conductances as function of tyrocidine B concentration both sides, in 1 mM potassium phosphate, pH 7.2; temperature, 36.5°. +, soya bean lipid[1]; ⊙, 1:1 molar soya bean lipid–cholesterol; ●, chromatographically pure plant phosphatidyl choline; □, chromatographically pure egg lecithin; △, bacterial phosphatidyl ethanolamine[3].

Fig. 2. Conductance development as function of time, linear plot. Lipids: 1:1 molar soya bean lipid–cholesterol. Tyrocidine concentration, $1 \cdot 10^{-8}$ M. Transmembrane potential, 20 mV.

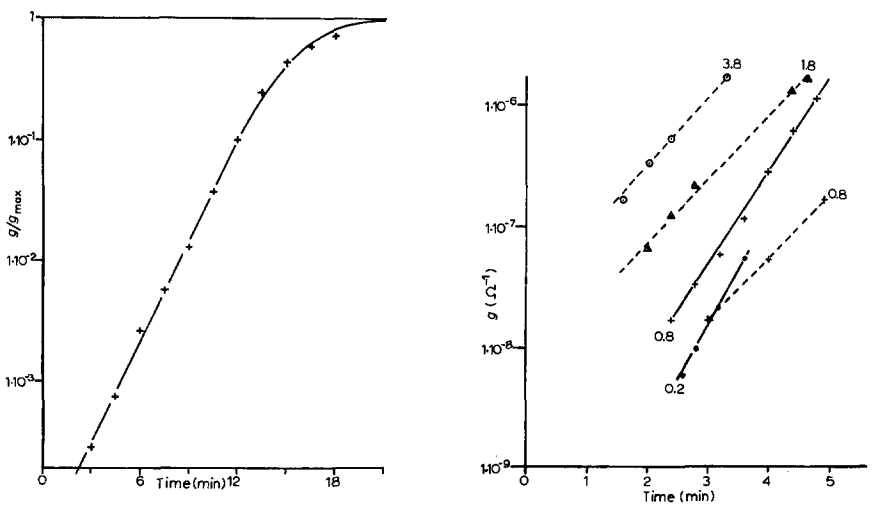

Fig. 3. Log–linear plot of the results of Fig. 2, compiled from a continuous record over 4 decades. Solid line, the function $\frac{1}{2}$ [1 + Tanh $k_1^+ (t-t_0)$].

Fig. 4. Same plot as Fig. 3, showing independence of autocatalytic rate k_1^+, from the parameter, concentration (× 10^{-7}). ———, soya bean lipid; — — —, 1:1 molar soya bean lipid–cholesterol.

$\frac{1}{2}[1 + \text{Tanh}\, k_1^+ (t - t_0)]$ and this verified by the log-linear plot of Fig. 3 which also exhibits the measurable dynamic range. From this plot one extracts two constants, a rate k and latency parameter $\mu = \exp[-kt_0]$ which are the slope and intercept of the linear portion.

Fig. 4 shows that these parameters k_1^+, μ (the latter for equilibrium conductance normalized to 1) are virtually independent, *i.e.* $\text{dlog}k/\text{dlog}a < 0.2$, of antibiotic concentration in the range $1 \cdot 10^{-9}$–$1 \cdot 10^{-7}$ M. The presence of cholesterol in the bilayer gives a smaller rate and a notably larger parameter μ. Fig. 5 shows that k_1^+ has a positive temperature coefficient indicating an activation heat of nearly 20 kcal; μ on the other hand, has unexpectedly a large negative temperature dependence.

Fig. 6 shows, in a linear plot, the field effect on the initial rate: a 7 times increase for a potential difference increment of 60 mV, while the subsequent exponential rate remains unchanged.

These results, with minimal interpretation, are summarized schematically as follows: let G be a "conducting complex" derived from the antibiotic A

$$G \rightleftarrows 2A + M \text{ (fast reaction)} \tag{1}$$

so that, conductance $\sim$(antibiotic)^2 (factor); and let the rate limiting kinetics of the "factor" M be

$$\frac{dm}{dt} = k^+(m + m^*) - k^- m^2 \text{ (autocatalytic)} \tag{2}$$

where "starting factor" concentration $m^* = \mu\bar{m}\, e^{eV/kT}$, with $\bar{m} = k^+/k^-$. Then the numerical values are,

$$\mu = \begin{matrix} 0.7 \\ 1.7 \end{matrix} \times 10^{-5},\ k^+ = \left.\begin{matrix} 0.03 \\ 0.02 \end{matrix}\right\} \text{sec}^{-1} \text{ for } \left\{\begin{matrix} \text{PL} \\ \text{PLC} \end{matrix}\right. \text{at } 37°$$

$$\Delta H_1^+ = 16\,000 \text{ kcals (PL)}$$

where PL is soya bean lipid and PLC is 1:1 molar soya bean lipid–cholesterol. To proceed further with interpretation of this factor, two things need to be taken into account; independence of concentration and the existence of field dependent "starting factor". This can be explained by saturation adsorption and a direct slow reaction respectively. Let X be special bilayer sites occupied by A with kinetics given by

$$\frac{dx}{dt} = k_0\{a(a_0 - x) - a_0 x\} \text{ (fast adsorption)} \tag{3}$$

so that effectively

$$x = \frac{a\, a_0}{a + a_0} \text{ (adsorption isotherm)} \tag{4}$$

Let M be formed by two pathways (a molecular interpretation of these will be given in the discussion)

$$X + M \underset{k_1^-}{\overset{k_1^+}{\rightleftharpoons}} 2M \text{ (rate limiting autocatalytic)} \tag{5}$$

$$X \underset{k_2^-}{\overset{k_2^+}{\rightleftharpoons}} M \text{ (slow) with } \frac{k_1^+}{k_1^-} = \frac{k_2^+}{k_2^-} = \frac{\bar{m}}{a_0} \tag{6}$$

Then

$$\frac{dm}{dt} = k_2^+ + (k_1^+ x - k_2^-)m - k_1^- m^2 \tag{7}$$

Threshold for autocatalytic reaction

$$a > \frac{a_0 k_2^-}{a_0 k_1^+ - k_2^-} \sim a_0 \mu e^{-eV/kT} \tag{8}$$

With

$$m^* = \frac{k_2^+ x}{k_1^+ x - k_2^-} \tag{9}$$

The above can be further tested by two experiments designed to show the independence of Reaction 1 from 2. Fig. 7, Curve B, shows the effect of preincubation with a low ($1 \cdot 10^{-8}$ M) concentration of antibiotics as compared with control, Curve A, where the final concentration ($1 \cdot 10^{-6}$ M) is added immediately. As predicted preincubation greatly speeds up the subsequent development of conductance. A similar and somewhat sharper result is obtained if the preincubation is done with the final concentration of antibiotic but in the absence of salt. On addition of the latter the development of conductance is immediate.

Another test depends on the pH dependence of Reaction 1. Fig. 8A shows that when pH on one side is lowered to 4 the conductance is lowered, but only when potential is such as to drive H^+ into the bilayer. Since the sweep frequency (0.06 sec^{-1}) is 3 times the rate constant k^+ of Reaction 5, and the effect is not frequency dependent and shows no phase shift in this range, one concludes that Reaction 1 is indeed a faster reaction than 5.

Apart from the effect of field and cholesterol on Reaction 6 further evidence of

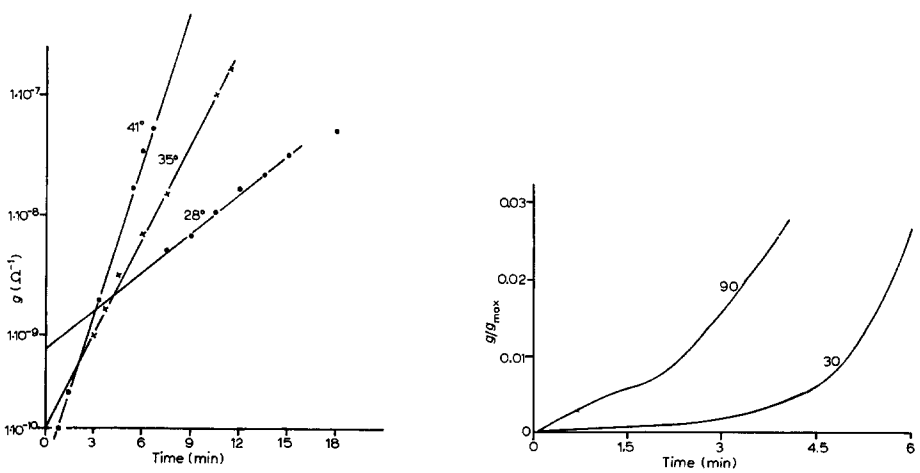

Fig. 5. Showing temperature dependence of autocatalytic rate; lipid: soya bean lipid. Tyrocidine concentration, $1 \cdot 10^{-8}$ M.

Fig. 6. Linear plot of initial development of conductance, showing effect of the parameter, transmembrane potential, in mV. Lipid: soya bean lipid. Tyrocidine concentration, $1 \cdot 10^{-7}$ M.

nature of Reactions 5, 6 comes from behavior in the one sided presence of antibiotic. Fig. 9 shows that in this case the rate constant k_1^+ of Reaction 5 is not only very much decreased (10-fold) but is now field dependent, although independent of its polarity. The behavior of the latency parameter indicates that the rate constant k_2^+ of Reaction 6 is even further decreased (100-fold) but is now almost independent of

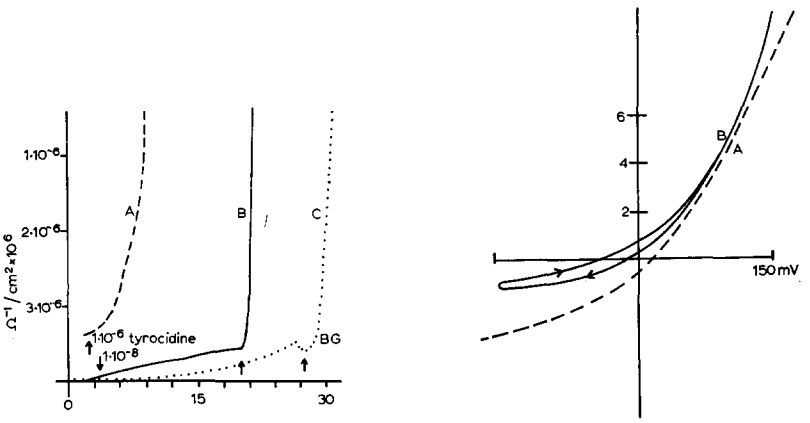

Fig. 7. Linear plot of conductance development. A, control; B and C, preincubated at low ($1 \cdot 10^{-8}$ M) concentration of tyrocidine B, ↑ further addition of $1 \cdot 10^{-6}$ M, ↑ addition of 0.5 mg/ml brain ganglioside (BG; as solid). Lipids: for A and B, bacterial phosphatidyl ethanolamine; for C, chromatographically pure egg lecithin. Transmembrane potential: 100 mV for A and B, 30 mV for C.

Fig. 8. Current–voltage plots. A, pH lowered to 4 by addition of HCl, ground side only; B, 1 mM $CaCl_2$ added to ground side only; negative potential is due to sodium phosphate in place of potassium phosphate on ground side, the accompanying Goldman type rectification was small, however. Sweep frequency $f = 0.06$ sec^{-1}. Ordinate (in A): A, $\times 10^8$; B, $\times 10^9$. Lipid: soya bean lipid.

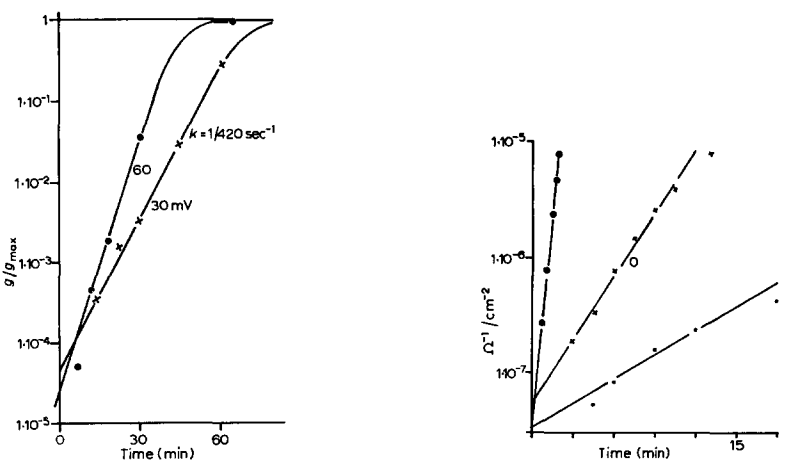

Fig. 9. Field effect on autocatalytic rate k_1^+ when $1 \cdot 10^{-7}$ M tyrocidine is added on ground side only. Potential is positive. Lipid: chromatographically pure plant phosphatidyl choline.

Fig. 10. Charge profile effect on autocatalytic rate k_1^+. ×, control; ⊙, brain ganglioside (0.5 mg/ml) both sides; ●, 1 mM $CaCl_2$ both sides. In each case the standard 1 mM potassium phosphate is present. Tyrocidine concentration, $1 \cdot 10^{-7}$ M. Lipid: chromatographically pure plant phosphatidyl choline.

field. The equilibrium conductances follow k_1^+ with approximate proportionality showing that Reaction 1 does not have a "sideness" dependence.

Finally there are some results showing that both Reactions 1 and 5 are markedly dependent on charge profile of the bilayer. First there is the result already noted in Fig. 1 that a lipid, which in the absence of antibiotic shows a marked anion selectivity (P_{Cl}/P_K approx. 100) and therefore presumably having a positive surface charge, shows a greatly decreased (350-fold) conductance with antibiotic, and also an appreciable (3.5-fold) reduction in autocatalytic rate.

This effect of charge profile can be demonstrated in one and the same lipid by agents added to the aqueous phase which adsorb onto the bilayer surface. Two such agents are Ca^{2+} and brain ganglioside giving +, — surface charge respectively, the latter because of terminal neuraminic acid residues.

It has previously been observed bare phosphatidyl choline bilayers will adsorb Ca^{2+} as evidenced by a transient spike of potential when placed on one side. Fig. 8B shows the effect of this surface charge in the presence of antibiotic; when the potential is such as to desorb the Ca^{2+} the conductance is very much increased. This can be shown directly by adding $CaCl_2$ (1 mM) to both sides after equilibrium conductance has been reached, there is an immediate order of magnitude decrease with only minimal stirring.

The converse result for brain ganglioside is shown in Fig. 7C. The effect of each agent on the autocatalytic rate k_1^+ is shown in Fig. 10. An interesting point here is that the "starting factor" of Eqn. 2 is not affected.

DISCUSSION

One can put these observations together into a scheme of molecular events shown in Fig. 11. If the half-saturation values are assumed to satisfy

$$a_0(Na^+) < a_0(K^+) \qquad (10)$$

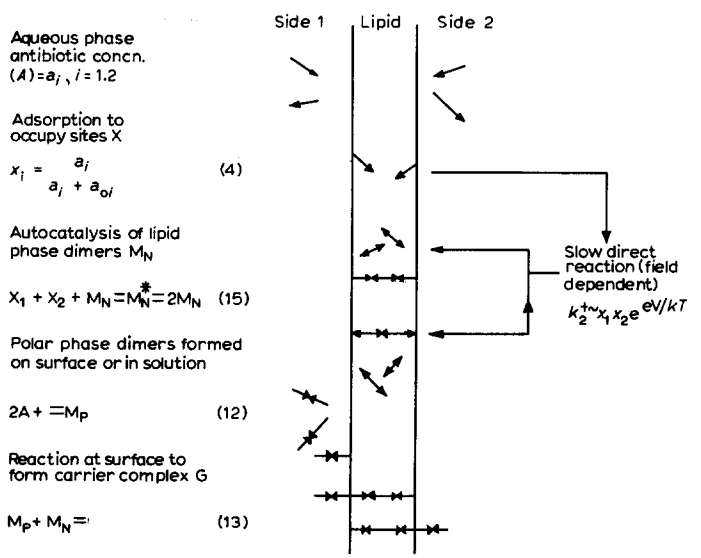

Fig. 11. Molecular events in the action of tyrocidine on lipid bilayer.

then Eqn. 12 explains the anomalous result[1] that the threshold for development of Na^+–K^+ bionic potential appears at a lower concentration of antibiotic when this is on the Na^+ side only as against the K^+ side. A further requirement for the result of Fig. 9 is that

$$a_0(V) < a_0(0) \tag{11}$$

To get a further interpretation of Reactions 1 and 5, the basic idea is to consider them as involving two kinds of dimerisation associated with polar surface and lipid phase represented by the species M_P, M_N, respectively. For this purpose Eqn. 1 should be broken down into

$$2A \rightleftarrows M_P \tag{12}$$

followed by

$$M_N + M_P \rightleftarrows G \tag{13}$$

and Eqn. 5 interpreted as two sided lipid phase dimerisation

$$X_1 + X_2 \rightleftarrows M_N \tag{14}$$

and Eqn. 5 accordingly

$$X_1 + X_2 + M_N \rightleftarrows \overset{*}{M_N} \rightleftarrows 2M_N \tag{15}$$

However, the following observation shows that the species M_N may not be freely dispersed as a solute in the hydrophobic region as in a bulk phase. For, if after reaching equilibrium conductance (t approx. 20 min), the solutions are vigorously stirred then the conductance falls by about an order of magnitude and after cessation of stirring recovers at the previous rate at that conductance. By observations one can see that the effect of stirring is to mix some of the bulk phase of the rim into the black region since a small proportion of thick parts also appear as bright specks. Therefore the term transmembrane structures appears advisable for M_N.

The existence of polar and non-polar configurations for monomer cyclic depsipeptides has been established by OVCHINNIKOV *et al.*[4]. A similar result for cyclic polyethers has been found by MCLAUGHLIN *et al.*[5]. The precise mechanism of Reaction 13, which might be expected to exhibit the ion interference effects[1] previously observed and the charge dependent effects observed here, must depend on knowledge of the tertiary structure of tyrocidine B.

In I the possibility that "tyrocidine" is effectively contaminated by the structurally related gramicidin S (ref. 6) was ruled out. That gramicidin A is a contaminant was not at the time considered (owing to the confusing nomenclature). However, the demonstration[7] that the latter gives quantised conduction fluctuations is consistent with the "push-on" or "guided carrier" mechanism depicted in Fig. 11. This and the question of lipid specificity, already evident in Fig. 1, will be considered in more detail in a following paper. Here one notes simply that for a small peptide, Reaction 6 becomes plausible with some compression of the bilayer, as would in fact be produced by electric field or cholesterol[8].

For technical reasons, of low conductance and long times, Eqn. 11 would be difficult to check directly. Indirect evidence, however, comes from results[9] obtained with two other cyclic peptides, alamethicin and monamycin. In both these cases the

kinetics of conductance development is considerably faster in K^+ as compared to Na^+ solutions. The conductance, however, is not selective for these cations, so that their action is not on a carrier mechanism. In the case of monamycin, it is known that it complexes with K^+ more strongly than Na^+, at least on the surface of bacterial cells[10].

In conclusion the action of "tyrocidine" involves the formation and interaction of polar and non-polar phase dimers. In this respect it occupies a place intermediate between the simple neutral carriers (valinomycin, enniation B, macrolide actins) and the highly associative antibiotics (gramicidin S, monamycin, alamethecin, monazomycin, nystatin and amphotericin B) which in the latter cases may be considered "pore forming"[11].

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REFERENCES

1 M. C. GOODALL, *Biochim. Biophys. Acta*, 203 (1970) 28.
2 G. SZABO, G. EISENMAN AND S. CIANI, *J. Membrane Biol.*, 1 (1969) 346.
3 J. G. HILDERBRAND AND J. H. LAW, *Biochemistry*, 3 (1964) 1304.
4 YU. A. OVCHINNIKOV, V.T. IVANOV, A.V. EVSTRATOV, V. F. BYSTROV, N. D. ABDULLAEV, E.M. POPOV, G. M. LIPKIND, S. F. ARKHIPOVA, E. S. EFREMOV AND M. M. SHEMYAKIN, *Biochem. Biophys. Res. Commun.*, 37 (1969) 668.
5 S. G. A. MCLAUGHLIN, G. SZABO, G. EISENMAN AND S. CIANI, *Biophys. Soc. Abstr.*, (1970) 96.
6 A. STERN, W. GIBBONS AND L. C. CRAIG, *Proc. Natl. Acad. Sci. U.S.*, 61 (1968) 734.
7 S. B. HLADKY AND D. A. HAYDON, *Nature*, 225 (1970) 451.
8 S. OHKI, *Biophys. J.*, 9 (1969) 1195.
9 M. C. GOODALL, *Nature*, 225 (1970) 1257.
10 M. J. HALL, *Biochem. Biophys. Res. Commun.*, 38 (1970) 590.
11 V. W. DENNIS, N. W. STEAD AND T. E. ANDREOLI, *J. Gen. Physiol.*, 55 (1970) 375.