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

III. GRAMICIDINS "A" AND "S", AND LIPID SPECIFICITY

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SUMMARY

1. Gramicidins "A" and "S" differ not only structurally but, in their action on bilayers, functionally in at least four respects: kinetics, overall order of reaction, ion selectivity and ion dependence of kinetics. In each case, Gramicidin "S" behaves more like a Tyrocidine (and is, therefore, here renamed Tyrocidine "S").

2. In mixtures (Tyrothricin), Tyrocidine inhibits the action of Gramicidin "A" and changes its kinetics to that approaching the "autocatalytic" behavior of Tyrocidine itself.

3. Tyrocidines, but not Gramicidin, show ion-dependent kinetics with rates increasing in the order $K^+ < Na^+ < NH_4^+$.

4. The above actions are marked by lipid specificities, which do not depend on net charge but rather on the presence or absence of amino groups: the former give 2–3 orders of magnitude lower conductance with either antibiotic, and autocatalytic behavior with Gramicidin "A".

5. The presence of cholesterol (2:1 mole ratio to phospholipid) reduces the Gramicidin conductance by over an order of magnitude while leaving that of Tyrocidine unchanged.

6. The different actions of Gramicidin and Tyrocidine with respect to ions accounts for some of the properties of the mixture, Tyrothricin. Their complimentary behavior can be interpreted in terms of a common molecular mechanism, previously proposed for the latter, the essence of which is a distinction between polar and nonpolar reactions, the latter generally speaking being rate limiting.

INTRODUCTION

Gramicidins "A" and "S" are not structurally related (see Fig. 8) and the reason for considering them together here is their relation to the Tyrocidines. The latter are produced by *Bacillus brevis* together with some 20 % of Gramicidin A, and the mixture as such was named Tyrothricin by HOTCHKISS AND DUBOS[1]. Thus, unless purified by

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countercurrent distribution, Tyrocidine preparations invariably contain Gramicidin A. As will be shown hereunder, if the actions of these were independent, a contamination by the latter of only 0.01 % would be sufficient to dominate the action of such Tyrocidine. However, it turns out that Tyrocidine has an inhibitory action on Gramicidin A and, therefore, a somewhat higher fraction of the latter must be present to manifest such contamination.

The results of the previous studies[2,3] of what was thought to be Tyrocidine "B" can now be seen to exhibit this contamination to the extent of some 0.4 % and henceforth this material will be referred to as "Tyrothricin". Gramicidin S on the other hand is structurally related to the Tyrocidines and, as will be shown, behaves similarly to purified samples of the latter. It will, therefore, be included in the generic term "Tyrocidines".

In the previous study[3], of this "Tyrothricin" lipid specificities were looked for and found unexpectedly to depend more on the phospholipid than the presence or absence of cholesterol. Furthermore, this specificity could not be explained by net charge differences or even apparently by charge configuration of polar groups. Therefore, and in view of the above clarification, these relationships are further investigated here, as well as the possible mode of interaction between the components of Tyrothricin.

These relationships are especially important in elucidating the mechanisms of action which, as pointed out[3], depend on the proper assignment of roles to polar and non-polar reactions between antibiotic monomers.

METHODS

These were as described previously[2,3]. Gramicidin A (Nutritional Biochemicals) was used as purchased. Owing to its great potency and necessary dilution, a problem can be presented by the adsorption of material to surfaces. To avoid this a dilution series (in methanol) was made repeatedly in the same vials and used freshly. To remove contamination from the Teflon chamber a wash of hot caustic soda followed by HNO_2 and then hydrazine was used. The effectiveness of this wash was tested by checking for absence of Na^+–K^+ bionic potential normally found in presence of Gramicidin A. Tyrocidines (A, C, S) were pure by countercurrent distribution[4]. As previously all measurements were made in 1 mM potassium phosphate (pH 7.2) at 36.5° with a polarising potential of 60 mV, unless otherwise stated.

RESULTS

Table I shows a comparison of the principal measurements for Gramicidin and Tyrocidines. From its overall order of reaction, which is six, and Na^+ selectivity, it is seen that Tyrocidine S is correctly classified as such and the differences from A and C can be attributed mainly to the extra (positively) charged ornithine group on the former (see Fig. 8). Another decisive difference between Tyrocidines and Gramicidin is the absence of sidedness effects for the latter, *i.e.* its activity is about the same whether placed on one or both sides of the bilayer.

The overall action of Gramicidin A is second order (Fig. 1) as also the kinetics (Fig. 2) in phosphatidyl choline bilayers, with rate constant $k = 6.7 \cdot 10^{-4}$ sec^{-1}. From

TABLE I

COMPARISON OF IONIC CONDUCTANCE PARAMETERS FOR GRAMICIDIN AND TYROCIDINES

n, overall order of reaction. n^*, apparent order of rate-limiting reaction, with rate constant k^*. σ I, σ II, equilibrium conductances with antibiotic one side, both sides. PL, phospholipid. PLC, phospholipid/cholesterol (1:1 mole ratio). PI, phosphatidyl inositol. PS, phosphatidyl serine.

Antibiotic	n	n^*	ΔH^* (*kcal*)	$k^*(Na^+)/k^*(K^+)$	$kT d\log k^*/edV$	VK^+–Na^+	VNH_4^+–Na^+	$\alpha = \sigma I/\sigma II$	$\sigma PL/\sigma PLC$	$\sigma PI/\sigma PS$
Gramicidin	2	2 0 (PS)	0	1	~ 0	+ 45	+ 35	1	20	$1 \cdot 10^3$
Tyrocidine A, C	6	0	20	4	1	− 8	+ 35	$< 1 \cdot 10^{-2}$	1	$1 \cdot 10^3$
Tyrocidine S	6	2	~ 30	6	2	− 15				

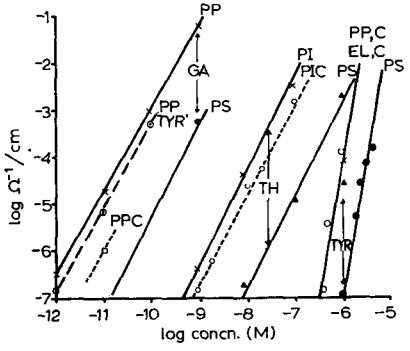


Fig. 1. Equilibrium conductance as function of antibiotic concentration, GA, Gramicidin A; TH "Tyrothricin"; TYR, Tyrocidine. Lipids: PP, EL, plant and egg phosphatidyl choline; PI, phosphatidyl inositol; PS, phosphatidyl serine; PEA, bacterial phosphatidyl ethanolamine. Followed by C, cholesterol, 1:1 mole ratio. "TYR", in the presence of a constant $3 \cdot 10^{-7}$ M Tyrocidine C.

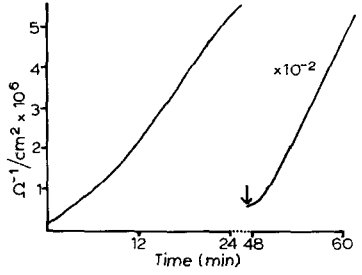


Fig. 2. Kinetics of conductance development in presence of Gramicidin A. Initial concentration $1 \cdot 10^{-11}$ M. ↓ increased to $1 \cdot 10^{-10}$ M, with stirring at 3-min intervals. Lipid, plant phosphatidyl choline. At ↓ ordinate scale is adjusted by the factor indicated.

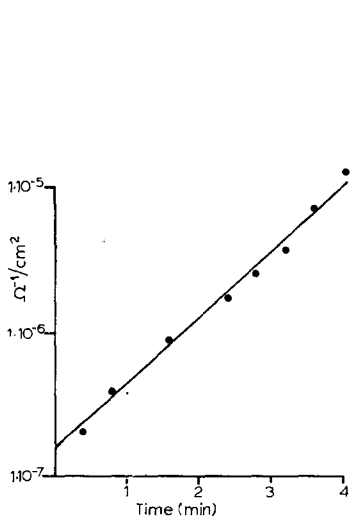
Fig. 1 it can be seen that the presence of 0.4 % Gramicidin A in the Tyrothricin mixture would be sufficient to account for its conductance. However, the fact that the kinetics of the latter is autocatalytic[3] shows that there must be some interaction.

This interaction shows first of all in a reduction of equilibrium conductance by the addition of a threshold concentration of Tyrocidine to Gramicidin, which by itself would give barely measurable increment of conductance; a result which becomes more intelligible when one makes a detailed comparison of kinetics particularly in relation to lipid specificity.

Fig. 3 shows that with phosphatidyl serine in place of phosphatidyl choline which from Fig. 1 gives two orders less equilibrium conductance, the kinetics become autocatalytic with rate constant, k approx. $2 \cdot 10^{-2}$ sec^{-1}, about the same as that found for Tyrothricin[3]. By the analysis given there, one can estimate the rate constant for the slow direct reaction to be approx. $1 \cdot 10^{-6}$ sec^{-1}.

Fig. 4 shows that Tyrocidine A gives strictly autocatalytic behavior with kinetics closely fitting the hyperbolic tangent function, as found for Tyrothricin[3]; the rate constant, $k = 1.2 \cdot 10^{-2}$ sec^{-1}, is somewhat smaller than for the latter which may be related to the fact that the equilibrium conductance is also smaller than for Tyrocidine C (Tyrocidine B might be expected to lie between, see Fig. 8). Tyrocidine S, however shows kinetics with less than exponential rate of increase. Although this might be associated with stirring artifacts it was found quite consistently and is probably real. The form of the curve suggests a log–log plot which is given in Fig. 5.

This plot shows that the kinetics of Tyrocidine S is described initially by a power law with an exponent of approx. 3; it also shows a field effect on rate which, in contrast to Tyrocidines A and C, for which it is zero, is quite large and is represented by a shift on the log (time) axis by an order of magnitude for a change of 30 mV in potential



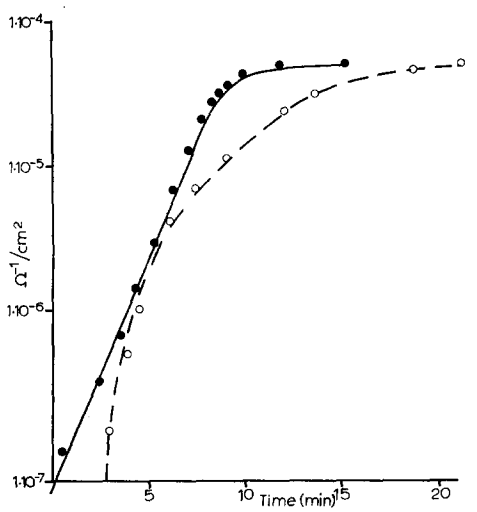


Fig. 3. Autocatalytic action of Gramicidin ($1 \cdot 10^{-9}$ M) on phosphatidyl serine bilayer.

Fig. 4. Comparison of kinetics for Tyrocidines A (●) and S (○). Concentration, $1 \cdot 10^{-6}$ M. Lipids, phosphatidyl choline (plant), and ditto (egg) with cholesterol, respectively. Difference between lipids is not significant.

difference. Extrapolation to zero field gives a rate constant $k\ (0) = 1.5 \cdot 10^{-4}\ \text{sec}^{-1}$ assuming overall kinetics of the form $(1-e^{-kt})^s$ with $s = 3$.

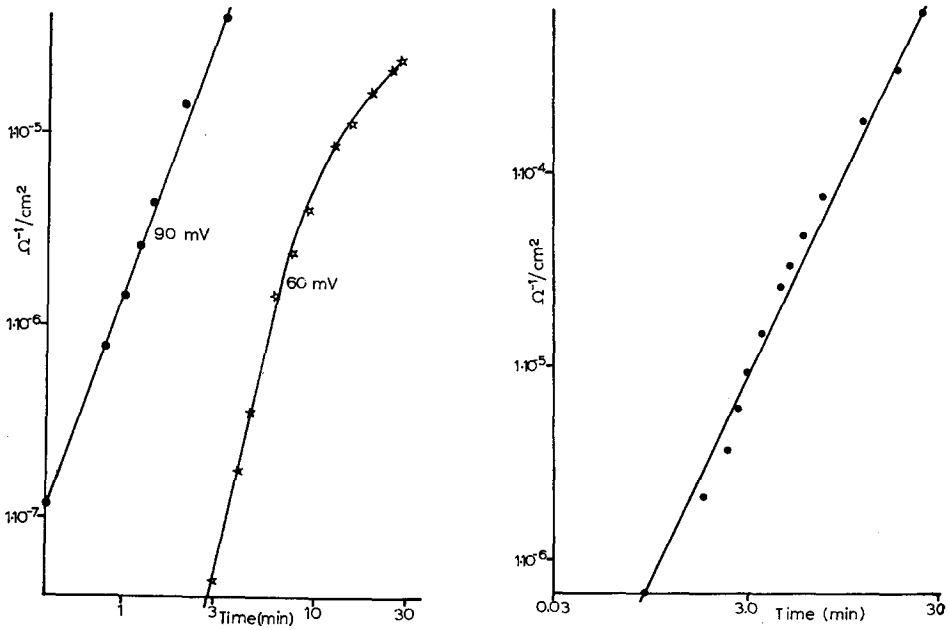


Fig. 5. Tyrocidine S as in Fig. 4, but with two different polarising potentials and on a log–log plot.

Fig. 6. Gramicidin ($1 \cdot 10^{-10}$ M) in the presence of a threshold ($3 \cdot 10^{-7}$ M) concentration of Tyrocidine C, same plot as Fig. 5. Lipid, plant phosphatidyl choline.

Fig. 6 shows the kinetics obtained with Gramicidin in the presence of a threshold (*i.e.* itself producing a barely observable increment of conductance) concentration of Tyrocidine. This now turns out to be "power law" with a slope of 2 and rate constant of $k = 5.5 \cdot 10^{-4}\ \text{sec}^{-1}$, *i.e.* about the same order of magnitude as Tyrocidine S at zero field.

Tyrocidines but not Gramicidin show ion-dependent kinetics. One way of showing this is to set up a bionic situation, then according to the polarity of the potential difference applied to measure conductance, the kinetics will be dominated by one or other ion. Fig. 7 shows the result of such an experiment with Na^+–K^+ and Tyrocidine

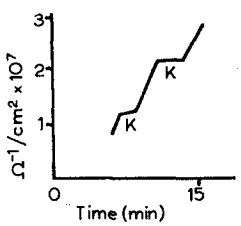
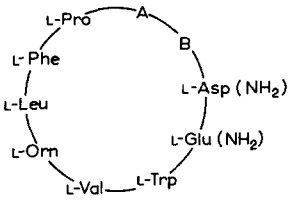
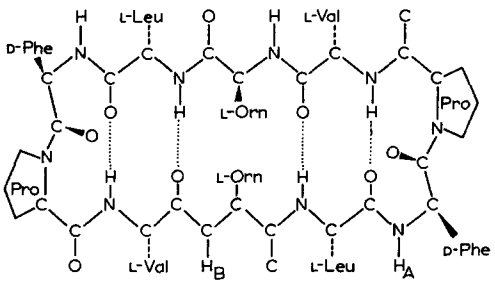


Fig. 7. Initial conductance development with Tyrocidine S, as function of polarity in a bionic Na^+–K^+ situation. Intervals marked "K" indicate that this ion is entering the bilayer. Lipid, plant phosphatidyl choline. Tyrocidine C gave a similar result. Gramicidin showed no effect.

C. In cases where there is appreciable biionic potential (*i.e.* Na^+–NH_4^+) and field effect (*i.e.* Tyrocidine S) these must be separated; the purely ionic effects in these cases, however, were somewhat greater. They will be the subject of further study.



Tyrocidines A–D



Tyrocidines S

HCO—Val—Gly—Ala—D-Leu—Ala—D-Val—Val—D-Val—Trp
D-Leu—Trp—D-Leu—Trp—D-Leu—Trp—NH_2 CH_2—CH_2—OH

Gramicidin A

Fig. 8. Amino acid sequence of Tyrocidines A–D; the positions AB are occupied by L–D amino acid pairs phenylalanine–phenylalanine, tryptophan–phenylalanine, tryptophan–tryptophan, phenylalanine–tryptophan, respectively. For Tyrocidine S the left half of the above sequence is repeated cyclically, the tertiary structure is shown below. Gramicidin A has a strict DL sequence since glycine is ambivalent.

DISCUSSION

In spite of the clearly characterisable differences between Gramicidin and Tyrocidines it seems probable that their mode of action is similar and can be described by a suitable generalization of the scheme proposed for Tyrothricin[3]. In this scheme conductance g was written as

$$g = a^n m \qquad (1)$$

the product of two factors, the first representing the product of a fast nth-order reaction and the second the product of a zero-order rate-limiting reaction which may be straight or autocatalytic or a mixture of these (two pathways). The zero order of the latter was attributed to saturation adsorption. If however the first factor is rate limiting, then there are various possibilities of which the following appears appropriate: a

rate-limiting pth order reaction followed by a fast sth order reaction with, $sp = n$. In this case we have

$$g = a^n(1 - e^{-kt})^s \quad (2)$$

where k is the limiting rate constant. If $s>1$ one has power law kinetics. Thus if n^* is the order of the rate-limiting reaction, we have $n^* = p$ or 0. In the latter case the kinetics are autocatalytic, since the other possibility, straight zero-order kinetics for m, is equivalent to $n^* = p = n$. The values of n^* estimated in this way are shown in Table I.

In the case of Gramicidin, where $n = 2$ and all the three possible forms of kinetics, $n^* = 0, 1, 2$, are found, departure from the straight case, $n^* = n$, appears to be associated with the introduction of charge. For positive (Tyrocidine) and negative (phosphatidyl serine) charge, the rates respectively of the first and second factor are inhibited, giving $n^* = 1, 0$. The presence however of power law kinetics, $n^* = 1$, with Gramicidin inhibited by Tyrocidine, is not consistent with the results obtained with Tyrothricin[2], where $n^* = 0$. The origin of this difference is not as yet clear, though there was some evidence that this may be caused by a trace contaminant since autocatalytic behavior was sometimes observed with Gramicidin alone in phosphatidyl choline bilayers.

On the other hand for the Tyrocidines alone the effect of net charge on the antibiotic shows up in high activation energy and field effects on the rate constants. The presence of an extra positive charge on Tyrocidine S again shows in power law kinetics.

The lipid specificity is remarkable, since it indicates that, unlike the case of neutral carriers[5], net charge of lipid is not the decisive factor as far as equilibrium conductance is concerned, but rather the presence or absence of amino groups: that is phosphatidyl choline and inositol give about the same higher conductance on the one hand as compared with phosphatidyl ethanolamine and serine on the other, while each of these pairs give about the same in spite of the net charge difference. The role of amino groups here may be hydrogen bonding to some part of the antibiotic molecule. This lipid specificity has little effect on rate constants in the autocatalytic case, in contrast to the effect of surface charge which was found to be considerable[3]. This difference is intelligible in terms of the latter affecting the saturation adsorption concentration for the second factor in Eqn. 1. On the other hand GOLDMAN[6] has proposed a theory of excitable membranes in which the polar ends of phospholipids change their orientation and combining properties under an electric field. This would indicate that in the above not merely net charge but charge structure is important. It was tentatively proposed[3] that the factors in Eqn. 1 be associated respectively with polar and no-polar reactions. It is satisfactory then that field effects, involving charge structure, should apply to the former.

These relationships need to be worked out in terms of the structures exhibited in Fig. 8. Gramicidin, a linear molecule, with possibly hindrance to cyclisation, could be formed into a cyclic dimer (with antisymmetry) in a hydrophobic environment by hydrogen bonding between the terminal formyl and ethanolamine groups. The D–L sequence, a notable feature of this and some other surface-active peptides would favor configuration of β-turns[7]. This is also a feature of Tyrocidine S where in addition the cross hydrogen bonding constrains it to a bar shape, characteristic of highly associative antibiotics, as opposed to the open ring structure typical of neutral carriers[8]. In view of

the evidence from quantised conduction[9], something like this must be true of Gramicidin, it could then have a somewhat longer bar shape, spanning the hydrophobic region of the bilayer, and this would favor the straight reaction for factor m referred to above.

Finally the ion dependence of kinetics of Tyrocidines presents a first step toward accounting for the ion-interference effects observed in a previous study[2] since NH_4^+ might be expected to increase Na^+-conductance mediated by Tyrocidine relative to the K^+-conductance mediated by Gramicidin. The complimentary behavior of the antibiotics with respect to these ions invites the speculation that their occurence together in the organism may serve the role of a regulation mechanism. In any case it is remarkable that the reaction scheme based on Eqns. 1 and 2 is, with $p = 1$, formally similar to the phenomological description of the Na^+–K^+ conductances in nerve by HODGKIN AND HUXLEY[10], including the field effects on the rate constants.

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