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INTERACTION OF ANTIBIOTICS WITH MEMBRANES: POLYMYXIN B AND GRAMICIDIN S

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SUMMARY

The interaction of the peptide antibiotics polymyxin B and gramicidin S with the lecithin–water model membrane system have been studied. Techniques used include Differential Scanning Calorimetry, ESR and NMR spectroscopy, ultraviolet and infrared spectroscopy, optical rotatory dispersion and ultracentrifugation. The results indicate that polymyxin interacts with both the polar and nonpolar region of the phospholipid, whereas gramicidin S interacts only with the polar region. The conformation of the antibiotics is not changed during the interaction. Gramicidin S solubilizes synthetic dipalmitoyl lecithin in water, the resulting particles still appear to contain tightly packed lipid chains in a bilayer state. The implication of these observations with respect to lipid–protein interactions and antibiotic action is discussed.

INTRODUCTION

In the present paper we extend our studies of the interaction of antibiotics and membranes[1] to the action of the peptide antibiotics polymyxin B and gramicidin S using lipid–water systems as a model membrane system. Polymyxins are thought to interact with the phosphate group of phospholipids, but apart from earlier monolayer studies[2] no investigation using modern physical techniques has been made to check this idea. There also exists some confusion concerning the question as to whether polymyxin interacts with lecithin or only with phospholipids bearing a negative charge. Attempts to relate the resistance of Proteus to polymyxin with the degree of methylation of the ethanolamine group in phospholipids[3] have failed, and liposomes prepared from lecithin are disrupted by polymyxin.

Gramicidin S has been extensively studied as far as structure[4], conformation[5] and biosynthesis[6] are concerned, but very little is known about its action on bacteria. It has been previously shown that gramicidin S is able to solubilize lecithin dispersed in water[7]. This causes a marked reduction in particle size and high resolution NMR signals are observed. As well as for the understanding of antibiotic action this interaction between a peptide and a phospholipid is of general interst for the discussion of lipid–protein interaction in membrane structure.

MATERIALS AND METHODS

Gramicidin S was a product of Mann Research Laboratories, England. Polymyxin B sulphate was a product of Sigma, London and contained 8040 units/mg. Sources of lipids, preparation of samples and instruments used for differential scanning calorimetry, ESR spectroscopy, ultraviolet and infrared spectroscopy were the same as described in previous papers.

For NMR spectroscopy the samples were prepared in ^2H_2O or water and after mixing heated above the transition temperature of the lecithin for a short time. Proton NMR spectra were run on the 220 MHz instrument of the S.R.C. Service, ICI Runcorn, England. Phosphorus resonance was measured with a JEOL 60 MHz instrument with proton decoupling and CAT facilities.

For ultracentrifugation a Beckman Model E equipped with Schlieren optics was used and the samples run in distilled water at 20° (we thank Dr. S. Koppikar, Department of Biochemistry, University of Sheffield). Density measurement with a pyknometer and viscosity measurements using an Oswald viscosimeter were both done using a constant temperature bath at 20 ± 0.1°.

ORD spectra were run on a Fica Spectropol 1 spectropolarimeter. Samples were always prepared except where stated using synthetic dipalmitoyl lecithin.

RESULTS

Solubility properties of the antibotic and antibotic–lipid mixtures

Polymyxin B sulphate is readily soluble in water and when mixed with lecithin the turbidity of the dispersion decreases. When excess polymyxin is used (1 : 4) the solution even becomes optically clear if the initial concentration of polymyxin is chosen to be very high (around 30 %). This high initial concentration, necessary for complete solubilization of the lipid suggests a micellar type of arrangement. The concentration is such that it makes it unlikely that it is related to the biological action of this antibiotic. Gramicidin S itself is scarcely soluble in water at pH 7, and when salts (*e.g.* KCl) are added the antibiotic forms a gel. Gramicidin S after shielding the two positive charges at its hydrophilic site by the addition of ions (therefore diminishing the repulsive forces) appears to form aggregates involving the hydrophobic sites of the molecule. When gramicidin S is mixed with lecithin at ratios 1 : 1 or 2 : 1 at a concentration of 4 mg lipid per ml water the solution after heating the mixture for some minutes becomes optically clear. This clear solution also forms a gel after adding KCl, whereas the solution of polymyxin–lecithin is not sensitive to the addition of ions.

Differential scanning calorimetry

Polymyxin added to dipalmitoyl-lecithin in increasing amounts reduces the endothermic transition of the lipid which occurs at 42° and removes it completely at ratios 1:1 and 2:1. Gramicidin S does not remove the transition (Fig. 1) but shifts the transition temperature to lower temperatures. At equimolar ratios of lipid and antibiotic the transition is at 37°. Within the limit of error the amount of heat per mg lipid is unchanged.

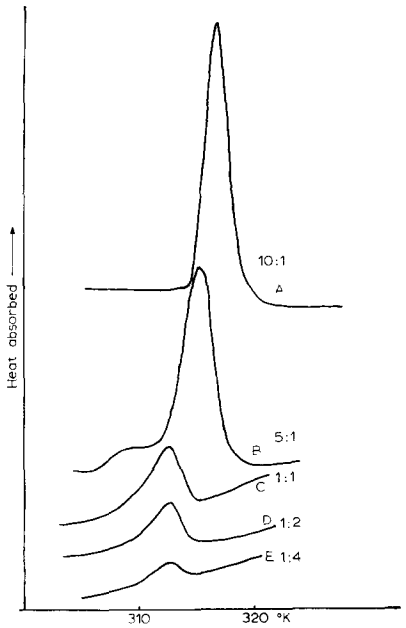

Fig. 1. Differential scanning calorimetry curves for lecithin–gramicidin S mixtures in water, compared with pure dipalmitoyl lecithin in water. The ratios shown are lecithin to gramicidin S.

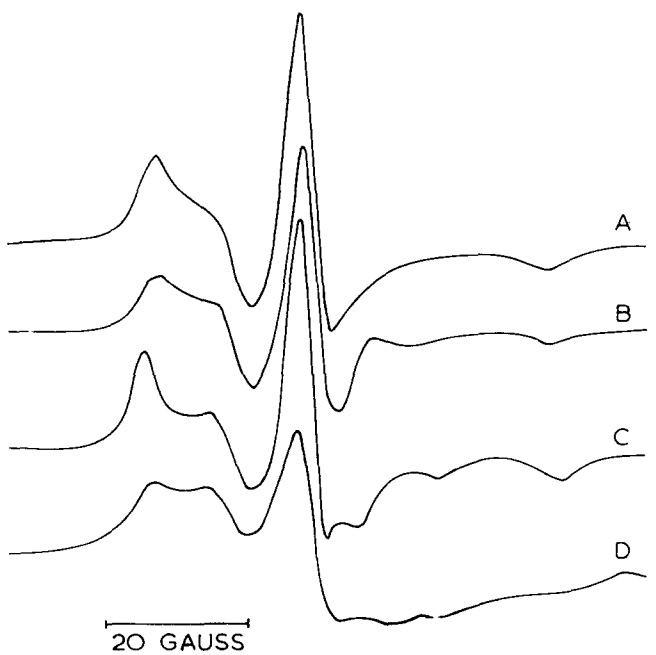

Fig. 2. ESR spectra of the 12-nitroxide methylstearate spin label in (A) dipalmitoyl lecithin, (B) dipalmitoyl lecithin–polymyxin B (1:1, w/w), (C) dipalmitoyl lecithin–gramicidin S (1:1, w/w), (D) dipalmitoyl lecithin–gramicidin S (1:2, w/w) in the optically clear solution.

ESR spectroscopy

When Polymyxin B or Gramicidin S is mixed with egg yolk lecithin containing the 12-nitroxide-methyl-stearate spin label (12 NS) the probe does not change its tumbling rate significantly. When synthetic dipalmitoyl-lecithin is used (below its transition temperature at room temperature) there is some increase in motion of the label when polymyxin is added (Figs. 2A and B). In contrast to this when gramicidin S is added to dipalmitoyl-lecithin the mixture still gives a strongly immobilized spin label spectrum. When the sample is diluted until it becomes optically clear the tumbling rate increases but the spectrum still looks fairly immobilized (Figs. 2C and D).

With cardiolipin and phosphatidylserine, negatively charged at pH 7 and with lipid chains in a fluid state at room temperature, gramicidin S and polymyxin B both give a precipitate but the spin probe does not change its mobility markedly.

NMR spectroscopy

The proton resonance spectrum of the optically clear solution of gramicidin S–dipalmitoyl-lecithin is given in Fig. 3. At room temperature there is no signal from the lipid chains of the lecithin (the lipid is below its transition temperature) but a marked signal from the methyl-groups of the antibiotic is observed. No signal is observed from the $N^+(CH_3)_3$ protons of the lipid at room temperature, but it and the lipid chain $(CH_2)_n$ signal appear* when the sample is heated to 55°.

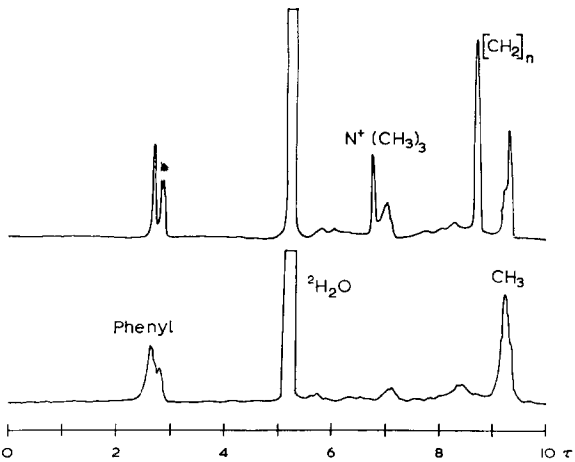

Fig. 3. 220-MHz spectra of dipalmitoyl lecithin–gramicidin S (1:2, w/w) in the clear solution in ^2H_2O. Lower spectrum at room temperature, upper spectrum at 55°.

With polymyxin the $N^+(CH_3)_3$ signal is absent at room temperature, but the change in intensity of the lipid chain signal by raising the temperature to 55° is not so pronounced as with gramicidin S.

The phosphorus resonance of the polymyxin–lecithin mixture (which can be prepared at sufficiently high concentrations) compared with lecithin in chloroform or a sonicated dispersion of egg yolk lecithin in water shows no shift.

* Signals from the $N^+(CH_3)_3$ group and the $[CH_2]_n$ group are observed in non-sonicated dipalmitoyl lecithin in ^2H_2O at 55°C but are much broader than occurs in this case.

Ultraviolet and infrared spectroscopy

The phenylalanine absorption of gramicidin S or polymyxin B around 260 nm is unchanged when egg yolk lecithin is present in a 5-fold excess of the antibiotics.

The infrared spectra of mixed films on AgCl plates show a shift in the P–O stretching frequency from 1250 to 1240 cm^{-1}. With cardiolipin (which forms a precipitate with the antibiotics) the shift occurs from 1240 to 1230 cm^{-1}. No shift in the absorption frequence of the amide bands of the antibiotics was observed.

ORD

The ORD spectra of gramicidin S and polymyxin B in water are similar to the spectra reported by CRAIG[8]. When lecithin is present no significant charge occurs in the minima and cross-over points.

Determination of the particle size in the gramicidin S–lecithin mixture

Ultracentrifuge runs of the optically clear solution af gramicidin S–lecithin show a single, rapidly spreading peak with a low S-value (Fig. 4). The S-values were found to be dependent on the time of heating the mixture and vary about a value of 2.5. For the calculation of an approximate molecular weight of the particles a typical value of $S = 2.7$ was chosen. The apparent partial specific volume for the solute was calculated from the density measurement of this solution and found to be 0.836. To obtain a value for the diffusion coefficient from an independent method D was calculated using the correlation time from the ESR spectrum (10^{-8} sec) and the viscosity of the solution (1.078 centipoise) from Stokes equation. It was found to be 10^{-6} $cm^2 \cdot sec^{-1}$. Using these data a molecular weight of 40000 was calculated for the particles.

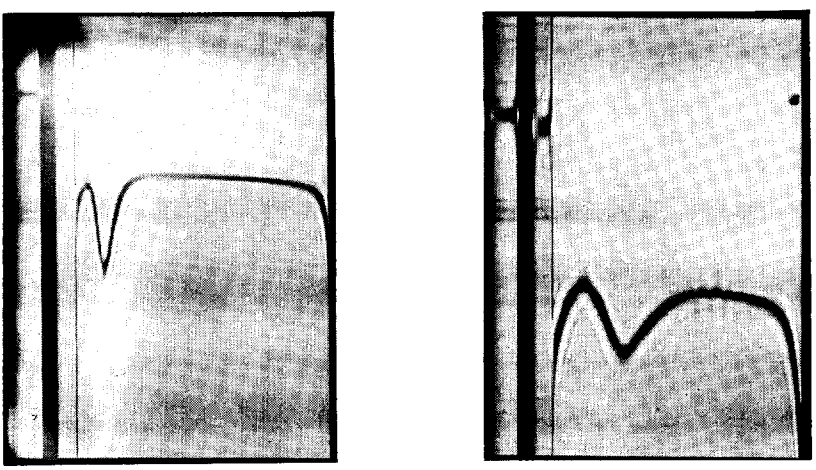

Fig. 4. Sedimentation peak of the dipalmitoyl lecithin–gramicidin S mixture (1:2, w/w) in the ultracentrifuge. Photographs taken (from left to right) after 0, 2 and 4 min after reaching speed.

DISCUSSION

There are two main questions which we pose: (a) What is the nature of the interaction between lipid and antibiotic, electrostatic or hydrophobic or both? (b) What relevance have these model experiments to the action of these antibiotics on cell membranes and to the interaction between lipids and proteins involved in the structure of cell membranes?

Nature of lipid-antibiotic interaction

Polymyxin B contains a branched fatty acid attached to the end of the side chain[4]. The complete removal of the endothermic transition of the lipid at 1:1 ratio is consistent with a penetration of the lipid chains by the branched tail of the polymyxin[9]. This shows that polymyxin is involved in a non-polar interaction.

This suggestion is confirmed by ESR and NMR spectra. When polymyxin is mixed with dipalmitoyl-lecithin a medium state of fluidity is achieved (Fig. 2) as indicated by the use of methyl stearate spin label. However, evidence for an involvement of the polar group is obtained from the small shift of the P–O stretching frequency observed in the infrared spectrum. This shift is also observed when polymyxin interacts with a negatively charged phospholipid, *e.g.* cardiolipin. As can be seen from the infrared absorption of the peptide region and the ORD spectra in the presence of lecithin during this interaction the conformation of the peptide ring of the antibiotic is not changed.

We conclude that in the case of polymyxin the interaction takes place by an electrostatic interaction between the amino-groups of the antibiotic and the phosphate group of the lecithin, and that the fatty acid tail of the polymyxin penetrates into the lipid bilayer. The peptide ring itself seems not be to incorporated into the lipid.

With gramicidin S the situation is somewhat different. The same statements are valid as far as electrostatic interaction (infrared spectra) and conformation of the antibiotic (infrared and ORD spectra) are concerned. However, the gramicidin S contains no fatty acid side-chain which could penetrate into the lipid chain region of the bilayer, the endothermic transition is not removed and both ESR an NMR spectra show tightly packed lipid chains at room temperature. The NMR spectrum of the gramicidin S–lecithin mixture shows signals from the phenyl groups as well as from the valine and leucine side chains at room temperature when the lipid signal is absent, thus confirming the view that the peptide ring is not incorporated into the lipid region. The sensitivity of the gramicidin S–lecithin mixture to the addition of ions is an indication that the interaction is only electrostatic in nature, in contrast to the interaction between polymyxin and lecithin (see RESULTS).

Cell membrane and antibiotic action

Gramicidin S solubilizes lecithin, the resulting particles being of a molecular weight below 100000. The formation of these relatively small particles must be due to the remaining positive charges on the gramicidin S–lecithin complex which tend to separate and therefore break up the bilayer. However, these particles do still contain regions of tightly packed lipid chains, since an endothermic transition is still observed when gramicidin S interacts with dipalmitoyl-lecithin although the transition is shifted to a lower temperature. In this respect gramicidin S clearly resembles a positively charged protein. Cytochrome *c* and lysozyme when interacting with phosphatidyl-serine also lower the transition temperature without causing a drastic change in the mobility of the lipid chains[10] and a similar mechanism is probably operating in the present case.

Our results are not directly related to the antibiotic action on the bacterial membrane, since these membranes do not contain lecithin[11]. However, with negatively charged phospholipids present (cardiolipin, phosphatidylglycerol, phosphatidylserine and phosphatidylethanolamine) the interaction is electrostatic and similar to the

interaction found in our model system. Polymyxin action for instance can be prevented by high ionic strength[12] and for both antibiotics the presence of free amino-groups is essential for microbial activity[4,13,14].

As pointed out for the case of the antibiotic chlorothricin[1] the minimum inhibitory concentration (m.i.c.) of the antibiotic should be kept in mind. With the reported m.i.c. of 10–100 μg/ml for gramicidin S[13] the ratio of antibiotic: lipid corresponds well to the ratio necessary for the interaction in the model system. In fact gramicidin S is necessary in equal or excess amounts to solubilize the lecithin, and there are indications that it is associated and therefore, behaves like a protein, especially in the presence of ions.

Polymyxin has a similar m.i.c. for gram-positive bacteria as gramicidin S, but with gram-negative bacteria the m.i.c. is much lower[13]. This selective action cannot be explained so far. There are suggestions that the target of the polymyxin in gram-negative bacteria is a lipoprotein complex of the cell envelope[11]. This does not rule out the possibility that the interaction between polymyxin and the lipid of this complex is the same as in our observations, but the greater sensitivity to the antibiotic makes it unlikely that the action is related to a simple bilayer of lipid.

When we consider the relevance of these lipid–polypeptide interactions to the nature of the lipid protein interactions involved in all membranes the manner in which gramicidin S is able to solubilize lecithin in water and transport it in an aqueous environment even when the lipid contains chains packed tightly together and in a bilayer form, is most interesting. It provokes the speculation that membrane proteins could similarly transport lipid to the site of membrane biosynthesis. The incorporation of the branched chain of polymyxin into the lipid bilayer may be analogous to the situation involved with the phytol chain of chlorophyll.

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