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INTERACTION OF ANTIBIOTICS WITH MEMBRANES: CHLOROTHRICIN

W. PACHE AND D. CHAPMAN

Department of Chemistry, University of Sheffield, Sheffield (England)

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SUMMARY

The mode of interaction of the antibiotic chlorothricin with cell membranes has been studied using lecithin–water dispersions as a model system. Various techniques have been used, *e.g.* monolayer and bilayer techniques, X-ray diffraction, differential scanning calorimetry, electron spin resonance, nuclear magnetic resonance, ultraviolet and infrared spectroscopy. At equimolar concentrations chlorothricin forms a strong complex with lecithin and prevents the phospholipid from swelling in water. The lytic action of the antibiotic can be understood in terms of a non-polar association of the antibiotic with the hydrocarbon chains of the phospholipid. Hydrogen bonding or ionic bonding is not an important factor in the interaction. Chlorothricin was shown not to transport ions across lipid bilayers.

Membrane phospholipids of *Bacillus subtilis* show a similar interaction. The effect of the chlorothricin in the model membrane is discussed and compared with the action of other antibiotics acting on membranes.

INTRODUCTION

Antibiotics have been shown to be useful tools for exploring biochemical processes in microorganisms, *e.g.* in the processes of cell wall synthesis and protein syntheses in bacteria. Antibiotics may also be useful for exploring membrane structure and function. Recent information of the mechanism of action of the ion transporting antibiotics for instance has already led to models for the transport of ions in cells[1,2].

An indication for an antibiotic affecting membranes is the binding to lipoproteins of egg yolk[3] or the antagonising effect of lecithin[4]. One of the antibiotics characterised in this way is chlorothricin[5], which has a definite action on the citric acid cycle and also lyses bacteria at concentrations slightly above the minimum inhibitory concentration (m.i.c.)[6]. Since the lecithin–water bilayer system is a well investigated example among the phospholipids[7] and there seems to be increasing evidence that some of the phospholipids of the cell membrane may be in a bilayer configuration[8–10], experiments were undertaken to see how chlorothricin interacts

Abbreviations: m.i.c., minimum inhibitory concentration; DSC, differential scanning calorimetry.

with lecithin and other phospholipids and to see how far its antibiotic action can be explained in these terms.

MATERIALS AND METHODS

Egg yolk lecithin was prepared according to the procedure of DAWSON[11]. Dipalmitoyl lecithin was purchased from Fluka, Buchs, Switzerland. Phosphatidylethanolamine was a product from Lipid Products, Epsom, Surrey, and Cardiolipin from Koch Light Laboratories, Colnbrook. Solvents used were analytical grade.

Monolayer studies were made with a conventional Langmuir trough with a torsion balance using waxed glass barriers. Distilled water from the same batch was used throughout all experiments. Samples were spread from hexane–ethanol (95:5 v/v) or, if chlorothricin was present, from benzene solutions.

Lipid bilayers were formed from a 1 % solution of egg yolk lethincin in decane. A teflon cup with a hole of 1 mm was used and the current measured with a Keithley 610 B Electrometer. Chlorothricin was added by dissolving it together with lecithin in chloroform and after evaporating the chloroform adding the decane.

Samples for differential scanning calorimetry (DSC), electron spin resonance (ESR) and nuclear magnetic resonance (NMR) spectroscopy were prepared by dissolving the lipid together with the antibiotic in chloroform and evaporating the chloroform using a stream of nitrogen. To remove traces of solvent, samples were held in a desiccator under high vacuum for at least 60 min. Then the water was added and the sample allowed to equilibrate for 30 min. For DSC the samples, which had a water content of 50 % were heated above the transition temperature of the synthetic lecithin. For ESR spin labels were added to the chloroform solution and the concentration in the final sample was chosen to be $2 \cdot 10^{-4}$ M. The lipid content of the final sample was 5 %. Rotational correlation times were calculated according to WAGGONER *et al.*[12]. For NMR spectroscopy the sample equilibrated with ^2H_2O was sonicated for 3 min whilst cooling with ice and blowing nitrogen over the sample The lipid concentration was 10 %.

Infrared spectra were run making a thin film of the samples in chloroform on NaCl plates and evaporating the solvent in a desiccator under vacuum.

The various instruments used for taking spectra were a Varian E 3 EPR X-band spectrometer, a Varian HA 100 NMR spectrometer, a Perkin Elmer 257 Grating infrared spectrophotometer and a Perkin Elmer DSC-1B differential scanning calormeter.

X-ray diffraction photographs were taken using a Rigaku-Denki low-angle diffraction camera with nickel-filtered Cu K radiation.

To obtain membrane lipids *Bacillus subtilis* Cohn (strain Tü 203 from the Institut für Mikrobiologie, Tübingen, Germany) was grown in two 8-l batches in a New Brunswick fermentation equipment for 16 h at 37° with an aeration of 2 l air per min, the medium used was the one described by BISHOP *et al.*[13]. Cells were harvested using a Sharples centrifuge, the membranes isolated by lysozyme–deoxyribonuclease (Koch–Light Laboratories) treatment and the lipids extracted from the freeze dried membranes with chloroform–methanol. The lipids were separated on a Silicar CC7 (Mallinckrodt, St. Louis) column following the procedure of BISHOP *et al.*[13].

RESULTS

Properties of chlorothricin and lecithin–chlorothricin mixtures

Chlorothricin with the approximate molecular weight of 950 contains one molecule of a 5-chloro-6-salicid acid, two molecules of a deoxy sugar and an aglycon of molecular weight 538 with a chromophore responsible for the ultraviolet absorption at 259 nm[5,14]. The antibiotic is a two-basic acid with pK's of 8.7 and 5.0. Whilst the free antibiotic is scarcely soluble in water but readily soluble in organic solvents of medium polarity, by preparing the sodium salt it is made water soluble. Mixtures of lecithin with chlorothricin at the ratios 1:1 and 2:1 exhibited the behaviour of a transparent viscous paste which does not mix with water. These mixtures dissolve completely in a solution of sodium dodecyl sulfate, but not in concentrated KCl or urea solutions. At lower concentrations of chlorothricin the mixture is able to swell with water.

Ultraviolet spectra

The only distinct absorption of the antibiotic is that of its sodium salt which has an absorption peak at 259 nm (log ε 4.16) when dissolved in water. Going to less polar solvents, short chain alcohols for instance, the peak disappears, and the remaining shoulder at 260 nm has a decreased intensity (log ε 3.90).

Chlorothricin itself scarcely dissolves in water of pH 6.5, but the small amount which dissolves shows the absorption peak typical for the dissociated form. By adding a suspension of phospholipid in water (2 mg/ml) to solid chlorothricin and stirring for 1 h at room temperature approximately a 2-fold amount of chlorothricin dissolves compared with the sample adding only water. The chlorothricin dissolved in the phospholipid suspension shows a spectrum with a shoulder at 260 nm.

Monolayer experiments

When chlorothricin as the sodium salt is added to water of pH 7.0 to give a concentration of 1 μg/ml (approximately 10^{-6} M), a rise in surface pressure occurs when the area is compressed to about 10 % of its initial size. Obviously some of the chlorothricin (probably undissociated) reaches the surface. The film starts collapsing at 17 dyne/cm, but since the amount of chlorothricin in the surface is not known, values for the area per molecule could not be calculated.

When chlorothricin (sodium salt, 1 μg/ml, pH 7.0) is injected below a monolayer of egg yolk lecithin compressed to 10 dyne/cm, the surface pressure increases to 18 dyne/cm. When the lecithin is compressed to 20 dyne/cm after adding the antibiotic no increase in surface pressure is observed.

At pH 3.0 (0.01 M citrate buffer), when chlorothricin is in the undissociated form, a monolayer of the antibiotic and a mixed film with lecithin can be obtained. The film of chlorothricin starts collapsing around 7 dyne/cm. At this pressure the area per molecule for chlorothricin is 180 Å^2. The mixed film of lecithin plus chlorothricin can be compressed to 10–12 dyne/cm before collapsing occurs.

Black film experiments

Black films formed from egg yolk lecithin in decane showed a resistance of 10^7–10^8 $\Omega \cdot cm^{-2}$ when measured in 0.1 M KCl solutions of pH 6.5. Chlorothricin,

when added up to a ratio of lecithin–chlorothricin of 10:1 (in decane) did not alter the resistance. When sodium acetate was used instead of KCl, the resistance was about one order of magnitude lower but chlorothricin again did not alter the resistance. (This latter experiment was undertaken because acetate interferes with the antibiotic action in the biological system.)

NMR spectroscopy

A sonicated dispersion of lecithin with chlorothricin at the ratio 10:1 in ^2H_2O shows a line broadening of the methylene protons peak but only a small broadening of the peak shown by the $N^+(CH_3)_3$ protons, the changes in half height line widths being 20→32 Hz and 5→6 Hz, respectively.

Differential scanning calorimetry

Chlorothricin added to synthetic dipalmitoyl lecithin at increasing amounts removed the endothermic transition step by step until it disappears completely at the ratio 1:1. At lower ratios no shift of the transition occurs (Fig. 1).

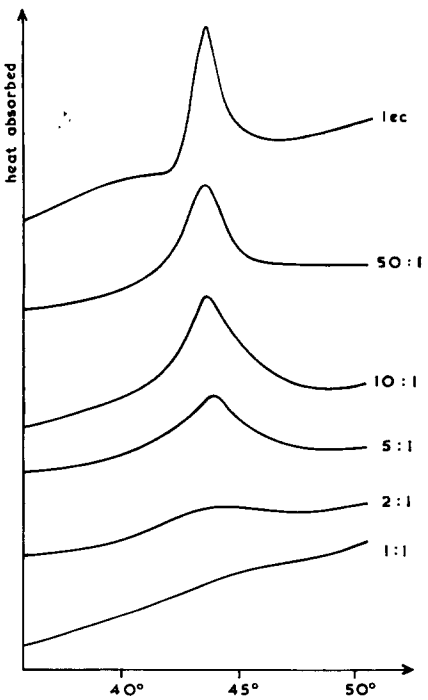

Fig. 1. Endothermic transition of dipalmitoyl lecithin and dipalmytoyl lecithin–chlorothricin mixtures. Ratios indicate the proportion of lecithin to antibiotic.

ESR spectroscopy

By using two different spin labels[15,16] (see Fig. 2) information about the state of the hydrocarbon chains as well as that of the polar group of the phospholipid in the lecithin–chlorothricin mixture was obtained. Fig. 3 indicates how the immobilization of the hydrocarbon chains increases by increasing the amount of chlorothricin in the mixture. In contrast, the nitroxide group of label II, which can rotate

around the single bond between the CH_2 and the nitroxide group is scarcely affected. Fig. 4 shows the ESR spectrum obtained with label I at a ratio of lecithin–chlorothricin of 2:1. The immobilization of the hydrocarbon chains can also be observed when using phosphatidylethanolamine and cardiolipin, two major phospholipids found in the membrane of grampositive bacteria.

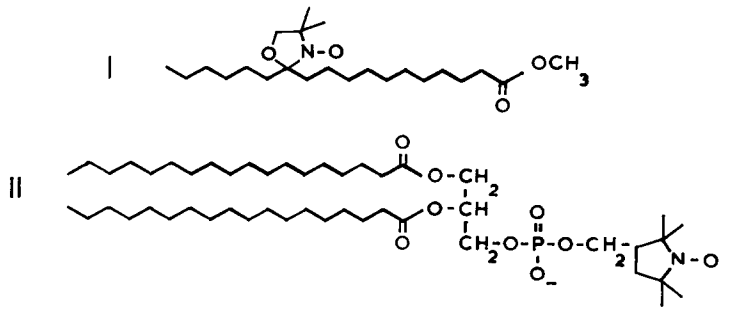

Fig. 2. Formula of spin labels. I, 12-(*N*-oxyl-4,4'-dimethyloxazolidine)-stearic acid methylester. II, *O*-(1',2'-distearoyl-*sn*-glycero-3'-phosphoryl)-3-hydroxymethyl-2,2,5,5-tetramethylpyrrolidine-1-oxyl.

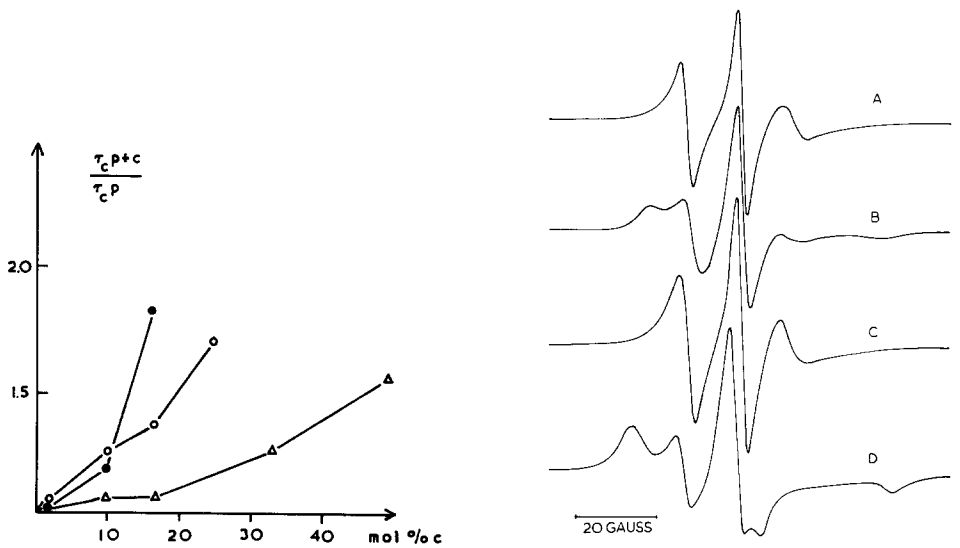

Fig. 3. Correlation times of spin labels in phospholipid–chlorothricin mixtures. Correlation times given are divided by the correlation time of the label in the respective phospholipid. ○, spin label I in egg yolk lecithin. ●, spin label I in phospholipids of *B. subtilis*. △, spin label II in egg yolk lecithin.

Fig. 4. ESR spectra of spin label I in A, egg yolk lecithin; B, egg yolk lecithin–chlorothricin, 2:1; C, phospholipids of *B. subtilis*; D, phospholipids of *B. subtilis*–chlorothricin, 2:1.

For the ESR experiments the methylstearate probe (I), which is known to orient less well than the corresponding free acid[17], had the advantage of showing isotropic motion at higher ratios of antibiotic:lipid than the free acid. Therefore correlation times could be calculated for a wider range (Fig. 3).

Infrared spectra

The only difference in the infrared spectra of the mixture lecithin–chlorothricin (1:1, v/v) compared with the spectra of the single components in a solid film, is the removal of the water peak of the phospholipid at 3400 cm^{-1} and a decrease in intensity of the 720 cm^{-1} band. No change either in the position of the OH-absorption of the chlorothricin or in the absorption of the phosphate group of the phospholipid at 1250 cm^{-1} occurs.

X-Ray diffraction

X-ray diffraction photographs of a dipalmitoyl lecithin–chlorothricin 1:1 mixture in the solid state show a diffuse diffraction at 31 Å only. At a ratio of 4:1 with 50 % water a lamellar phase is formed with diffractions corresponding to a spacing of 75 Å.

Interaction of chlorothricin with membrane lipids of B. subtilis

The yield of lipids from 50 g cells (wet wt.) was 400 mg. After separation the partition was 11.5 % neutral lipids, 5.5 % glycolipids and 82 % phospholipids, values are in good agreement with those reported by BISHOP *et al.*[13]. The yield of lipoamineacids (1 %) was too low for further experiments, and no polyhydroxybutyrate precipitate was obtained from the original extract.

DSC and ESR experiments were made with the total lipids and the single lipid classes. No transition was observed either for the total or for the phospholipids. A small but very broad transition occured with the neutral lipids, mainly diglyceride, around 10°. Fig. 3 gives the correlation times for the spin label 1 in the phospholipids in the mixture with chlorothricin, showing that the immobilizing effect of the chlorothricin is even more pronounced than with lecithin. An example of an ESR spectrum of bacterial phospholipid with added chlorothricin showing the motion of the spin label when it is no longer isotropic is given in Fig. 4. A decrease in mobility of the hydrocarbon chains is also observed in the mixtures of chlorothricin and glycolipids.

DISCUSSION

Lecithin dispersed in water has been extensively used as a model system for the cell membrane and as a result this system has been investigated in great detail. The main techniques which have been used are X-ray diffraction[7], calorimetry[18], NMR[19] and ESR spectroscopy[20], giving information about arrangement of the bilayer, heat transfer during the "melting" of the lipid chains, and fluidity, polarity and viscosity of the hydrocarbon region[21,22] respectively.

The properties of this lipid bilayer system are modified by other molecules present in membranes, *e.g.* protein or cholesterol, and also by antibiotics[23].

The questions we pose are (a) what is the nature of the interaction between the antibiotic chlorothricin and lecithin and (b) is this interaction similar to that which occurs with the bacterial membranes?

Chlorothricin–lecithin interactions

Let us consider the evidence concerning the first question.

The ultraviolet spectrum of chlorothricin in water and in a lecithin dispersion

shows that the spectral change (removal of the peak at 259 nm) is the same as when chlorothricin or its sodium salt is dissolved in less polar solvents.

The monolayer experiments demonstrate that the antibiotic enters a monolayer of lecithin when injected into the water at a concentration even lower than its minimum inhibitory concentration. The penetration of the monolayer is an indication for an interaction.

Although a lamellar phase is still present at lower concentrations of chlorothricin as shown by X-ray diffraction, an alteration in the spacing takes place. The antibiotic breaks up the lipid chain packing as confirmed by means of the calorimetric method. In removing the endothermic transition of dipalmitoyllecithin chlorothricin resembles cholesterol[24], but the interaction is nevertheless quite different. Lecithin solubilizes the cholesterol in water, whereas with chlorothricin a complex is formed at equimolar amounts of antibiotics and lecithin. Water is excluded from this complex and the hydrocarbon chains of the lipid are strongly immobilized both above and below the transition temperature of the lipid. X-ray studies show that in this dry state the packing of the lipid chains is affected.

The NMR studies which show selective broadening of the $(CH_2)_n$ signal, also provide additional evidence for the nonpolar interaction between lecithin and chlorothricin. The infrared spectrum showing the decrease in intensity of the 720 cm^{-1} band, associated with the all trans conformation of the methylene groups in the lipid chains is also an indication of an interaction involving the hydrocarbon chains.

The solubility properties of the equimolar complex of lecithin–chlorothricin in sodium dodecyl sufate solutions as compared with KCl or urea indicate that polar bonding, or hydrogen bonding, play if any at all only a minor part in the interaction. The spin label II which could indicate an interaction involving the polar group is not immobilized at lower ratios of chlorothricin–lecithin. Consistent with this the NMR experiments show only a minor effect of line broadening on the choline group signal.

Other facts consistent with this idea that hydrogen bonding is not an important factor in this interaction are (a) the OH-band of the chlorothricin is not shifted with regard to the chlorothricin itself and (b) there is no shift in the lecithin phosphate band in the infrared spectrum.

Chlorothricin–membrane interactions

Let us now consider the second question: does the antibiotic act on membranes in the same way as on lecithin? Chlorothricin is active against gram-positive bacteria growing on synthetic media. On complex media the activity of the antibiotic is reduced by the presence of lipids which might be free or associated with protein[5].

B. subtilis is affected by chlorothricin at a concentration of 4–5 μg/ml. Two major phospholipids of the lipids of this organism which were studied extensively by BISHOP *et al.*[13] and OP DEN KAMP *et al.*[25], are phosphatidylethanolamine and cardiolipin. These phospholipids show the same sort of interaction (*i.e.* using spin labels) with chlorothricin as lecithin. The total lipids as well as the different classes of lipids isolated from a culture of *B. subtilis* are also similarly affected (the spin-label was also immobilized with the glycolipids).

When chlorothricin interacts with phospholipids, the ratio of phospholipid to antibiotic at which a pronounced reduction in motion of the hydrocarbon chains

can already be observed is about 10:1. This seems a fairly high concentration of antibiotic, but considering the amount of lipid present in bacteria it becomes reasonable. From a 16-l culture of *B. subtilis* for instance, 400 mg of membrane lipids is obtained, 82 % of which are phospholipids. This corresponds to about 20 μg of phospholipids per ml culture, and with a concentration of 4 μg chlorothricin per ml (m.i.c.) the ratio is approx. 5:1 calculated for an average molecular weight of the phospholipids of 1000. This is well in the range when a pronounced effect is observed.

The lytic action of chlorothricin on bacteria can be explained by its interaction with the hydrocarbon chains of phospholipids. This interaction drastically reduces the fluidity of the lipid chains and since lipid chain fluidity seems to be an important condition for a functioning membrane it accounts well for permeability changes and distortion of the bilayer structure. Perhaps chlorothricin interacts with lipids bound to protein as well, resulting in a change in tertiary structure of the lipoprotein. Since membrane enzymes are known to depend upon phospholipids with their action often dependent on the presence of phospholipids[26], a change in lipoprotein structure caused by chlorothricin could have a lethal effect on the cell. The lack of specifity of the antibiotic with lipids suggests that an interaction with hydrophobic regions of proteins is also a possibility to be kept in mind.

A comparison of antibiotic action

It is interesting to compare the action of chlorthricin with the action of other antibiotics on model membranes. This shows that the different antibiotics have a different target (Fig. 5).

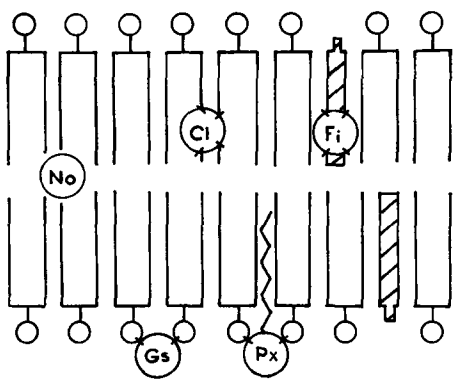

Fig. 5. Schematic picture of the interaction of different antiobiotics with a model membrane (lecithin and cholesterol). No, nonactin; Cl, chlorothricin; Fi, filipin; Gs, gramicidin S; Px, polymyxin B.

Polyene antibiotics are known for their specifity to steroids, and when added to a cholesterol containing lecithin bilayer, remove the sterol by forming a nonpolar association. In this way they change the fluidity of the lipid chains in the bilayer[27]. Gramicidin S associates with the polar region of the lecithin and solubilizes lecithin in water. Polymyxin interacts both with the polar and non-polar regions[27].

When ion transporting antibiotics (nonactin, valinomycin or boromycin) are mixed with lecithin, they separate from the lipid as soon as water is present and no change of the spin label in the lipid chains and no change in the endothermic

transition is observed (W. PACHE AND D. CHAPMAN, unpublished results). However, small amounts go into the lipid as can clearly be demonstrated by the change in resistance of black films[28]. SHEMYAKIN pointed out that their entering into the non-polar regions of membranes can simply be explained by their surface active properties alone[29]. In fact any tighter interaction with lipids would restrict their diffusion and therefore not favour the shuttle mechanism which has been suggested for these antibiotics[30]. Chlorothricin interacts still in a different way since it has an affinity for the hydrocarbon chains of the lipid.

The action of these various types of antibiotics on model membranes may well be related to their action on the membranes of microorganisms. The ion transporting antibiotics act at concentrations where an effect on the bilayer structure is unlikely (m.i.c. for nonactin, 0.05 μg/ml). All the other antibiotics mentioned act at concentrations about one or two orders of magnitude higher (m.i.c. for chlorothricin, 4–5 μg/ml), and at this concentration the amount of antibiotic is comparable with the amount if lipid present in the membrane. Therefore, the molecular details of the interaction with the lipid bilayer system are likely to be relevant to their *in vivo* antibiotic action.

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