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The interaction of penicillins with phospholipids

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The interaction of the antibiotics, penicillin G and ampicillin, with sonicated sols of phosphatidylcholine, lysophosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol and phosphatidylserine has been examined by Sephadex gel filtration and dynamic dialysis. Nuclear magnetic resonance spectroscopy provided evidence of a predominantly hydrophobic interaction between the antibiotics and the phospholipids, phosphatidylserine and phosphatidylcholine. Confirmation of hydrophobic interaction was provided by a rheological investigation of the effects of urea and guanidine hydrochloride on the antibiotic-phospholipid complex. Penicillin G was found to interact to a greater degree than ampicillin, a result which is of interest in the light of present knowledge of *in vivo* activity of these antibiotics.

While both penicillin G and ampicillin are active against Gram-positive bacteria, only ampicillin has any significant effect against Gram-negative bacteria. Gram-negative cell walls contain up to 26% of lipid whereas little or none is found in the walls of Gram-positive species (Salton, 1964); correlation between antibiotic resistance and cellular lipid, both in amount and composition, has been previously pointed out by Hugo & Stretton (1966), Dunnick & O'Leary (1970) and Biagi, Guerra & others (1970). We have examined the nature of the interaction of penicillin G and ampicillin with phospholipids. Although phosphatidylcholine and lysophosphatidylcholine are not found to any great extent in bacteria, they are useful models for a study of phospholipids since they have been well-characterized (Robinson, 1961; Attwood & Saunders, 1965; Saunders, 1966).

Interactions of phospholipids with cholesterol (Phillips, Kamat & Chapman, 1970), acetylcholine (Hauser, Phillips & Marchbanks, 1970) and polyene antibiotics (Finer, Hauser & Chapman, 1969), using nuclear magnetic resonance (Chapman & Penkett, 1966), monomolecular films (Demel, Crombag & others, 1968) and thermal analysis (Ladbrooke & Chapman, 1969) have indicated that the observed effects were due to a reduction in the configurational freedom and kinetic motions of the phospholipid hydrocarbon chains, the interactions being of a hydrophobic nature.

Studies on the interaction of penicillin G, and to a lesser extent ampicillin, have mainly concerned interaction with serum proteins to determine the amount of biologically-available antibiotic (Kunin, 1965), using nmr (Fischer & Jardetzky, 1965), and dialysis techniques (Scholtan & Schmid, 1962).

Acred, Brown & others (1963) reported a study of the protein-binding properties of the penicillins, studied by gel filtration and dialysis. They found that ampicillin, penicillin G and triphenylacetyl penicillin, differed in the degree and nature of their binding to serum proteins.

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MATERIALS AND METHODS

Materials. Potassium penicillin G was the gift of Glaxo Research Limited, Greenford. Penicillin G [^{14}C] was obtained from the Radiochemical Centre, Amersham. Sodium ampicillin and ampicillin [^{14}C] were the gifts of Beecham Research Laboratories, Betchworth. Phosphatidyl-L-serine (PS) and phosphatidylinositol (PI) were obtained from Koch-Light Laboratories Limited; all materials were chromatographically homogenous and were used as supplied.

99·95% deuterium oxide and tetramethylsilane were obtained from Fluorochem. Sephadex G-10 (40-120μm) and G-25 Fine (20-80μm) were used as filtration media. Analar urea (BDH) and Practical grade guanidine hydrochloride (Sigma) were used as supplied. Solvents were laboratory grade; glass-distilled water was used throughout.

Apparatus. An MSE 60W ultrasonic disintegrator was used to prepare the aqueous dispersions. All radioactive samples were counted on a Packard Tri-Carb liquid scintillation spectrometer, model 3380.

Nmr spectra were obtained at 31° using a Varian Associates HA-100 Spectrometer, operating in the field-sweep mode. A computer of average transients (CAT) (Varian Instruments Limited) was used to enhance the signal-to-noise ratio.

A Contraves AG Epprecht Rheomat RM-15-fc was linked to a Metrohm Labograph E478 for the rheological studies. The samples were placed in a concentric-cylinder film system (Type MS-O), capacity 4 ml, thermostatted at 25° $\pm$ 0·1°.

Preparation of phosphatidylcholine (PC). BDH 95/100% egg lecithin was purified by chromatography on an alumina column and eluting with chloroform–methanol, (6:1 v/v and 3:1 v/v). The PC fractions were identified by thin-layer chromatography on Kieselguhr G nach Stahl (Merck AG), using chloroform–methanol–water, (14:6:1 v/v). PC was recrystallized from warm methylethylketone by adding acetone; it was stored under acetone at –18°. Immediately before use it was checked for purity by thin-layer chromatography, the product giving a single spot, R_F 0·77 (1,2-dipalmitoylphosphatidylcholine, 0·76). N% 1·82 (literature values for egg PC, 1·78%).

Preparation of lysophosphatidylcholine (LPC). This was prepared by Saunders' (1957) modification of the method of Hanahan, Rodbell & Turner (1954), using the PC prepared in the previous method before recrystallization. The purity was checked by thin-layer chromatography on Kieselguhr G, the product giving a single spot, R_F 0·27 (1-palmitoyl-lysophosphatidylcholine, 0·27). N% 2·59 (literature values for egg LPC, 2·66%).

Preparation of phosphatidylethanolamine (PE). The method of Robins & Thomas (1963) was used, with the exception that Celite was added to the silicic acid column. The product was stored at –18°. Immediately before use the PE was checked for purity by thin-layer chromatography on Kieselguhr G, the product giving a single spot, R_F 0·88 (1,2-dipalmitoylphosphatidylethanolamine, 0·86). N% 1·84 (literature values for egg PE, 1·90%).

Gel filtration. Sols of PC, PE and PS were formed according to Attwood & Saunders (1965), with complete removal of ether before the sols were made up to weight.

Sols of LPC and PI were formed by directly adding water to a weighed quantity of the phospholipid and shaking until dispersed.

All sols were subjected to sonication in an atmosphere of nitrogen at 20 KHz for 90 min (Saunders, Perrin & Gammack, 1962). At the concentrations used, all of the phospholipids gave isotropic sols which were stable for at least 7 days.

Unless otherwise stated, antibiotic solutions at 0·5 mM concentration and phospholipid sols at 0·1 mM were used. Columns were prepared by adding a slurry of the swollen beads to a column 300 mm in length and 25 mm in diameter. The column was allowed to consolidate (24 h) before use when the G-25 Fine column had a bed volume of 80 ml. The antibiotic [^{14}C] -phospholipid mixture (1 ml) was carefully applied to the top of the column, and a constant head device was fitted to give an elution rate of 18 ml h^{-1}. 1 ml fractions were collected as soon as the antibiotic-phospholipid mixture had been added and the activity determined. The presence of phospholipid was tested by a standard molybdate test for phosphorus.

Dialysis. The dynamic technique of Meyer & Guttman (1970a,b) was used, the sac size and volume of sac solution being kept constant; 125 mm lengths of Visking tubing (18/32 pore size) and 5 ml of sac solution (0·5 mM [^{14}C] antibiotic–0·1 mM phospholipid) were used throughout. The sac was suspended from a clamp in 300 ml of water, thermostatted at 25°; the bath solution was stirred at 60 rev min^{-1}. Periodically 100 ml samples of the bath solution were removed and replaced by water. The radioactivity of an aliquot of each sample was determined. After 6 h the contents of the sac were mixed with the bath, and samples taken in order to determine the initial sac concentration.

Nuclear magnetic resonance spectroscopy. The phospholipid sols were prepared by adding D_2O directly to the weighed amount of phospholipid in a flask. Ether was not used to dissolve the phospholipid before addition of D_2O because it could not be obtained free from water. Nitrogen was bubbled through the coarse dispersion, which was then sonicated at 20 KHz for 10 min (Hauser, Penkett & Chapman, 1969).

The phospholipid-antibiotic mixtures were prepared by adding 0·5 ml of the sonicated phospholipid sol to the requisite amount of antibiotic. The mixtures were shaken to dissolve the antibiotic, and the spectra were obtained after 18 h.

Rheology. Various materials have previously been reported to act as structure-breakers; urea (Mukerjee & Ray, 1963; Davis, 1971) and guanidine hydrochloride (Schick & Gilbert, 1965) have been indicated as particularly effective. Studies were made using both these materials, to investigate the effect of concentrations up to 8M on the shear stress at a particular shear rate. 3/2 PC/LPC mixed sols were chosen for study as they had been shown previously (Padfield, 1972) to exhibit marked effects in the presence of the antibiotics.

RESULTS AND DISCUSSION

Although the frontal technique of gel filtration is more suited to quantitative studies of rapidly reacting systems of the type $A + B \rightleftharpoons C$, Hummel & Dreyer (1962) have demonstrated the feasibility of using the zonal technique to investigate interactions between proteins and small molecules. Sephadex G–25 Fine grade gave satisfactory separation of the peaks, whereas G-10 gave too rapid elution of the antibiotic. It has been reported for protein-antibiotic interaction (Acred & others,

Table 1. *Effect of antibiotic on the mol% of antibiotic bound to the phospholipids examined.*

	PC	LPC	PE	PI	PS
Penicillin G	100	100	100	100	100
Ampicillin	37·1	58·9	43·7	70·1	63·8

1963), that G-50 and G-75 grades lead to broadening of the protein peak and merging with the unbound-antibiotic peak.

Preliminary studies showed that elution with different concentrations (0 to 0·5 mM) of penicillin G did not alter the area of the bound-antibiotic peak. A contact time of 18 h was chosen for interaction to occur, as contact periods greater than 18 h gave no increase in the percentage of penicillin G bound to PC.

The molar percentage of bound antibiotic (calculated from the ratio of peak areas) in relation to phospholipids (Table 1) indicates that penicillin G is completely bound to all phospholipids at the 5:1 molar ratio studied. The amount of ampicillin binding depends on the nature of the phospholipid, the greatest effects being observed with the negatively-charged diacyl phospholipids (PI and PS) and least with the neutral zwitterionic diacyl phospholipids (PC and PE). These findings are in line with nmr studies on the interaction of phospholipids with adrenaline (Hammes & Tallman, 1971) and procaine and tetracaine hydrochlorides (Hauser & others, 1969).

Loss of the antibiotics from the dialysis sac is shown in Fig. 1 A and B. A least squares fitting procedure was used to plot the curves, most being well represented by a

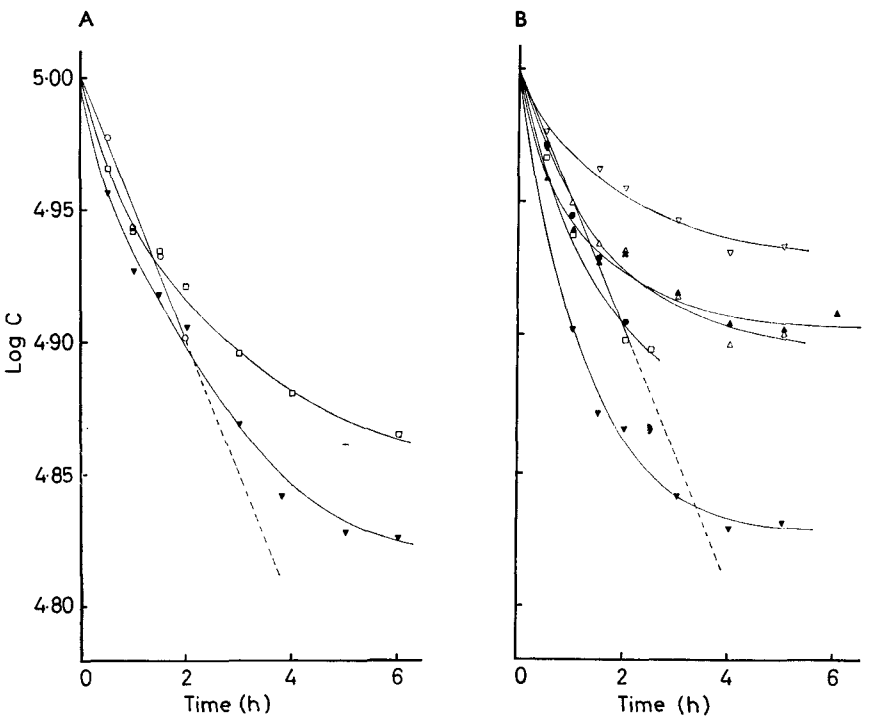

FIG. 1. A. Loss of penicillin G (○) from inside a dialysis sac at 25 ± 0.1° and in the presence of (▼) PC and (□) LPC.
B. Loss of ampicillin (●) from inside a dialysis sac at 25 ± 0.1° and in the presence of (▼) PC (□) LPC (△) PE (▽) PI and (▲) PS.

Table 2. *Effect of phospholipids on* 100 MHz *nmr signals of antibiotic protons in* D_2O *(τ values)* J *values in* Hz *in parentheses.*

Antibiotic-Phospholipid	C_6H_5-	α-CH-X[a]	C-6	C-5	C-3	C-2-$(CH_3)_2$	
Penicillin G	2·60	6·32	4·44d (4·0)	4·46d (4·0)	5·69	8·39	8·44
Penicillin G-PS[b]	2·53	N/O	N/O	N/O	N/O		8·44
Penicillin G-PC	2·48	6·19	4·43d (4·0)	4·47d (4·0)	5·65	8·31	8·39
Ampicillin	2·48	5·26	4·45s	4·45s	5·72	8·41	8·48
Ampicillin PS	2·47	c	4·47s	4·47	5·80	8·52	8·55
Ampicillin-PC	2·42	5·21	4·40s	4·40s	5·70	8·38	8·45

a. X = −H in Penicillin G. X = $-NH_2$ in ampicillin.
b. Values given are peaks of very broad signals. No signals observed for other groups.
c. Not observable under D_2O signal.
s = singlet.
d = doublet.

second degree polynomial expression, i.e., having a multiple correlation coefficient greater than 0·990. The ampicillin-phospholipid results are of the same order of effect as observed in Table 1, i.e. PI > PS > PE > PC. The rate of penicillin G transfer across the membrane was slower than ampicillin in the presence of the phospholipids (Fig. 1) and, as predicted by gel filtration was slower in the presence of LPC than PC.

Table 2 gives the chemical shifts for the antibiotics at 100 MHz; signal assignments are those of Green, Page & Staniforth (1965). The gel filtration data (Table 1) and the data of Hauser & others (1969) have indicated the suitability of PC and PS for nmr studies.

Figs 2 and 3 illustrate the addition of the antibiotics to PS; signal assignments being those of Chapman & Morrison (1966). The primary peaks observed (Figs 2a and 3a) are those due to the hydrocarbon chain, $(CH_2)_n$, protons and the chain terminal, CH_3, protons. Similar spectra were obtained for PC where, in addition, a strong peak due to the choline, $\overset{+}{N}(CH_3)_3$, protons was observed (Table 3).

Differential line broadening and line shifts were observed for some of the peaks on addition of penicillin G and ampicillin (Figs 2 and 3; Table 3). The penicillin G peaks most affected were those due to the aromatic and α -CH_2 groups (Table 2). The CAT spectra (Fig. 3b and c) indicate that the PC and PS peaks most affected were those due to the $(CH_2)_n$ and CH_3 groups (Table 3); the line broadening observed in the

Table 3. *Effect of antibiotics on (A)* 100-MHz *nmr signals (τ values) (B)* T_2 *relaxation times (s), of primary phospholipid protons in* D_2O.

	A.			B.		
Phospholipid-Antibiotic	-CH_3	-$(CH_2)_n$	-$\overset{+}{N}(CH_3)_3$	-CH_3	-$(CH_2)_n$	-$N(CH_3)_3$
PS	9·03	8·64	—	15·0	26·5	—
PS-Penicillin G	N/O	8·70	—	N/O	43·0	—
PS-Ampicillin	9·05	8·69	—	15·0	22·0	—
PC	9·16	8·66	6·66	22·0	30·0	28·0
PC-Penicillin G	N/O	N/O	6·70	N/O	N/O	17·0
PC-Ampicillin	9·08	8·62	6·62	17·0	20·5	6·0

N/O = not observable.

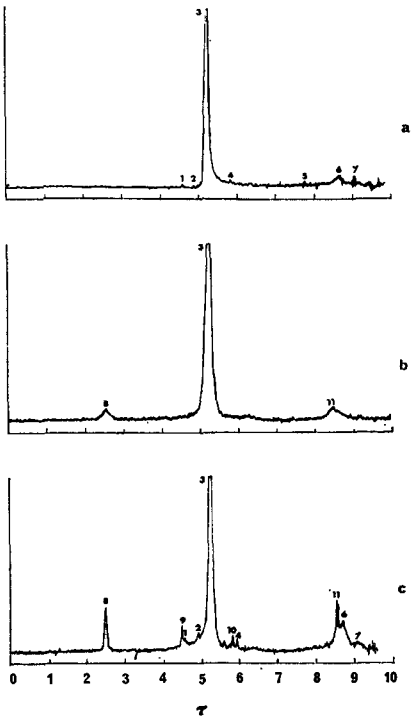

FIG. 2. 100-MHz nmr spectrum of 2% PS in D_2O in the presence and absence of the antibiotics (2%). a. PS (Peaks 1 to 7). b. PS + penicillin G. c. PS + ampicillin. 1. $-CH=CH-$. 2. $-CH-O-PO$. 3. D_2O. 4. $-CH_2-O-PO$. 5. $-CH_2-CO$. 6. $-(CH_2)_n$. 7. $-CH_3$. 8. C_6H_5. 9. $-CH-CH-$. 10. $-CH-COO^-$ 11. $-C(CH_3)CH_3$.

case of Penicillin G-PS was such that some of the signals were lost in the baseline (Fig. 2b).

We interpret the loss of the four peaks from the penicillin G spectra on addition of PS as a case of extreme line broadening, the peaks being lost in the baseline. In view of the observed changes in the PS spectra [$-(CH_2)_n$ and terminal $-CH_3$ signal broadening], it is difficult to envisage any form of interaction other than of a hydrophobic nature, which would thus implicate the aromatic ring and α-CH_2 group of the anti-

Table 4. *Effect of (A) urea (B) guanidine hydrochloride on % change in shear stress of 3/2 mixed PC/LPC sol (2% w/w) at a shear rate of 794 s^{-1} in the presence of antibiotics.*

Phospholipid-Antibiotic	0·5M	1M	2M	4M	8M
A.					
PC/LPC	+30·2	+37·9	+40·5	+67·2	−27·8
PC/LPC-Penicillin G	−2·0	—	−5·0	−27·2	−20·9
PC/LPC-Ampicillin	−14·3	−19·8	−25·0	−26·1	−43·0
B.					
PC/LPC	+8·3	+72·0	+143·5	+174·4	+118·2
PC/LPC-Penicillin G	+34·5	+5·6	−31·1	−44·1	−52·4
PC/LPC-Ampicillin	+46·2	+60·8	+43·6	+45·2	−38·0

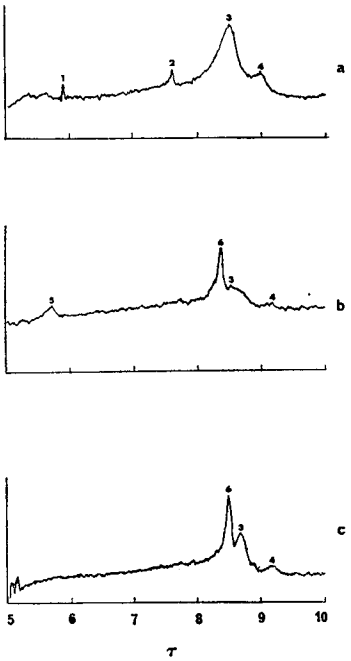

FIG. 3. 100-MHz nmr CAT-spectrum (15 scans) of 2% PS in D_2O in the presence and absence of the antibiotics (2%). a. PS (Peaks 1 to 4). b. PS + penicillin G. c. PS + ampicillin. 1. $-CH_2-O-PO$. 2. $-CH_2-CO$. 3. $-(CH_2)_n$ 4. $-CH_3$. 5. $-CH_2-$. 6. $-C(CH_3)CH_2$.

biotic. Confirmation of this view is provided by substituting PC for PS, when line shifts of 24 and 26 Hz were recorded for the aromatic ring and α-CH_2 group respectively (Table 2). Ampicillin, being less hydrophobic than penicillin G (Bird & Marshall, 1967), would be bound to a lesser extent; thus smaller changes were noted in the nmr spectra (Tables 2 and 3).

The effects observed on addition of the antibiotics to the phospholipids are similar to those observed on addition of cholesterol to phospholipids (Chapman & Penkett, 1966; Darke, Finer & others, 1972). These authors considered that the observed line broadening reflected a reduction in the configurational freedom and kinetic motion of the hydrocarbon chains.

Urea and guanidine hydrochloride are known to break hydrophobic bonds (Davis, 1971; Schick & Gilbert, 1965), and their addition to the phospholipid-antibiotic sols resulted in a decrease in viscosity at almost all concentrations (Table 4); the effect was greatest in sols to which penicillin G had been added. Decrease in viscosity is a reflection of structure breakdown in the system; the results indicate that these materials disrupt the hydrophobic interaction between antibiotic and phospholipid, giving a decreased viscosity.

Thus of the two antibiotics, penicillin G and ampicillin, the former interacts more strongly with phospholipids, and this observation we believe is significant in that penicillin G has little effect on Gram-negative organisms whose walls contain a large amount of lipid—little being present in Gram-positive organisms. It would suggest that, in interacting with the cell wall lipid, penicillin G is prevented from passing to

the cell membrane and reacting with the transpeptidase enzyme system involved in the synthesis of cell wall material. Ampicillin, in contrast, is involved to a lesser extent in interaction with bacterial lipid and can, therefore, pass through the cell wall and exert its effect at the membrane.

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