NMR Spectroscopy: Antibiotic Interactions with Phospholipids

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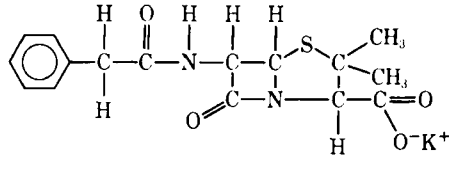
Abstract □ Sonicated dispersions of phosphatidylcholine, lysophosphatidylcholine, phosphatidylinositol, and phosphatidylserine in deuterium oxide were studied by NMR, and the changes in spectra produced on addition of penicillin G and ampicillin to the dispersions were observed. The antibiotics appeared to reduce the configurational freedom and kinetic motion of the phospholipid hydrocarbon chains due to hydrophobic interaction between the hydrocarbon chains and the phenyl portion of the antibiotic side chain. Penicillin G interacted with the phospholipid systems to a greater degree than ampicillin, while the phospholipids interacted in the order phosphatidylserine > phosphatidylcholine > phosphatidylinositol > lysophosphatidylcholine.

Keyphrases □ Antibiotic interactions with phospholipids—studied using NMR spectroscopy □ Phospholipids, interactions with penicillin G and ampicillin—studied using NMR spectroscopy □ Penicillin G and ampicillin interactions with phospholipids—studied using NMR spectroscopy □ Ampicillin and penicillin G interactions with phospholipids—studied using NMR spectroscopy □ NMR spectroscopy—used to study antibiotic interactions with phospholipids

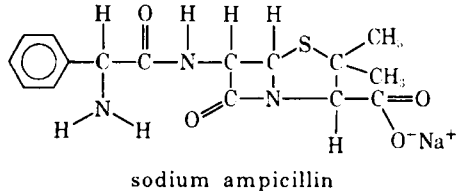
This work is part of a physical study (1) designed to investigate the reported differences in *in vivo* activity (2) of penicillin G (I) and ampicillin (II) by attempting to correlate these differences with the relative penicillin affinity for binding with phospholipids (III*a*, III*b*, III*c*, and III*d*). Previous work examined the effects of interactions on the rheology of phospholipid systems (3, 4), on diffusion of the antibiotics through phospholipid dispersions, and on gel filtration (5). The significance of such interactions was also discussed (5).

NMR spectroscopy is a useful method of investigating the relationships between lipids and other molecules because it often allows a single component to be studied in a heterogeneous environment (6, 7). Furthermore, Jardetzky (8) showed that NMR is invaluable in assessing the extent to which various functional groups on small drug molecules participate in drug–macromolecule interactions.

NMR spectroscopy has been used to study lipids (9–11) and lipid–water systems (12, 13), primarily by broad-line measurements. Sonication of the systems enabled a high resolution spectrum to be obtained (13–16) similar to that observed when the phospholipid was dissolved in a solvent such as deuterochloroform (17). Most workers indicated that the lamellar state is retained after sonication, *i.e.*, no phase change occurs, although this is not universally accepted (18).



potassium penicillin G
I



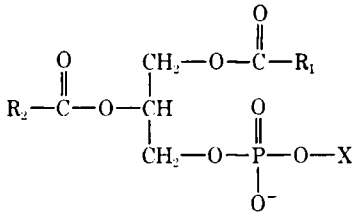
sodium ampicillin
II

These studies provide the basis for investigating the interaction of phospholipids with cholesterol (19, 20), acetylcholine (21), and polypeptide antibiotics (22, 23). The observed broadening of the lipid hydrocarbon chain proton signal has been interpreted as a reduction in the configurational freedom and kinetic motions of the hydrocarbon chains (19, 24). Further studies indicated binding of phospholipids to anesthetic molecules (25) and epinephrine (26).

NMR studies on penicillin G and ampicillin have not been widely reported. The demonstration of binding of penicillin G to serum albumin (27, 28) and guanosine (29) indicated the use of selective changes in relaxation times in assessing which portion of the penicillin molecule was involved in binding.

EXPERIMENTAL

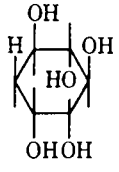
Materials[1]—Egg phosphatidylcholine (III*a*) was prepared as previously described (5). Highly purified lysophosphatidylcholine (III*b*), phosphatidylinositol (III*c*), and phosphatidylserine (III*d*)



III*a*: phosphatidylcholine, $X = -CH_2-CH_2-\overset{+}{N}(CH_3)_3$,
R_1 and R_2 = fatty acid chains (C_{13}-C_{23})

III*b*: lysophosphatidylcholine, $X = -CH_2-CH_2-\overset{+}{N}(CH_3)_3$,
R_1 = fatty acid chain (C_{13}-C_{17}, saturated), R_2 = H

III*c*: phosphatidylinositol, X =



R_1 and R_2 = fatty acid chains (C_{14}-C_{20})

III*d*: phosphatidylserine, $X = -CH_2-CH(NH_2)-COOH$,
R_1 and R_2 = fatty acid chains (C_{12}-C_{18})

[1] Potassium penicillin G was the gift of Glaxo Research Limited, Greenford, England, and sodium ampicillin was the gift of Beecham Research Laboratories, Betchworth, England; both materials were laboratory reference grade.

Table I—Effect of Phospholipids on 100-MHz. NMR Signals of Antibiotic Protons in Deuterium Oxide (τ Values) (J Values in Hertz in Parentheses)

Antibiotic–Phospholipid[a]	C_6H_5	α-CH—X[b]	C-6	C-5	C-3	C_2—$(CH_3)_2$		Remarks
Penicillin G	2.60	6.32	4.44d[c] (4.0)	4.46d (4.0)	5.69	8.39	8.44	Nothing downfield
Penicillin G–PC	2.48	6.19	4.43d (4.0)	4.47d (4.0)	5.65	8.31	8.39	
Penicillin G–LPC	2.53	6.24	4.39d (4.0)	4.43d (4.0)	5.70	8.36	8.43	
Penicillin G–PI	2.58	6.30	4.46d (4.0)	4.51d (4.0)	5.75	8.42	8.50	
Penicillin G–PS	2.53	—	—	—	—	8.44		Precipitation after 18 hr.[d]
Ampicillin	2.48	5.26	4.45s	4.45s	5.72	8.41	8.48	Nothing downfield
Ampicillin–PC	2.42	5.21	4.40s	4.40s	5.70	8.38	8.45	
Ampicillin–LPC	2.46	5.25	4.42s	4.42s	5.74	8.41	8.48	
Ampicillin–PI	2.51	5.21	4.50s	4.50s	5.80	8.50	8.56	
Ampicillin–PS	2.47	—[e]	4.47s	4.57s	5.80	8.52	8.55	

[a] Key: PC = phosphatidylcholine, LPC = lysophosphatidylcholine, PI = phosphatidylinositol, and PS = phosphatidylserine. [b] X = H in penicillin G, and X = NH_2 in ampicillin. [c] s = singlet and d = doublet. [d] Values given are peaks of very broad signals. No signals observed for other groups. [e] Not observable under deuterium oxide signal.

were obtained commercially[2]. The phospholipids were stored with a desiccant at −5°, and their purity was confirmed by TLC on kieselguhr G plates[3]. Deuterium oxide (D_2O), 99.95%, and tetramethylsilane were also used[4].

Preparation of Dispersions—The antibiotic solutions and phospholipid sols were 2% w/v unless otherwise stated. The phospholipid sols were prepared by adding deuterium oxide directly to the weighed amount of phospholipid in a flask. The dispersions were sonicated at 20 KHz. for 10 min. in nitrogen atmosphere (23, 25).

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 hr. (1).

NMR Measurements—The spectra were obtained at 31° using a spectrometer[5], operating in a field-sweep mode. The spectra of the phospholipids were accumulated on a computer of average transients[6]; 15 scans were normally sufficient. Tetramethylsilane provided a lock signal for the instrument and an external reference signal for measuring chemical shifts.

RESULTS

Table I gives the chemical shifts for the antibiotics in the 100-MHz. spectra; signal assignments are based on those of Fischer and Jardetzky (27) and Green *et al.* (30). Although a doublet was observed for each proton of the β-lactam ring in penicillin G, only one singlet was observed for both protons in ampicillin.

Figure 1 illustrates the high-resolution NMR spectrum of III*d* and its interaction products; the computer of average transients spectrum is shown in Fig. 2. Peak assignments are based on those of Chapman and Morrison (17). Table II indicates the chemical shifts observed for the phospholipids. The primary peaks observed in all spectra were those due to the hydrocarbon chain, $(CH_2)_n$, protons and the chain terminal, CH_3, protons; in addition, a strong signal was observed for the terminal choline, $^+N(CH_3)_3$, protons in III*a* and III*b*. These signals are only observed in sonicated dispersions, the fine structure being absent in unsonicated material (15, 16, 20). The $(CH_2)_n$ peaks, composed of more than 30 protons, are broader in appearance. Broad peaks are also characteristic of aggregate forms in dispersion.

The effect of the addition of penicillin G and ampicillin to sonicated dispersions of phosphatidylserine is indicated in Figs. 1*b*, 1*c*, 2*b*, and 2*c*. Differential line broadening and line shifts are observed for some peaks (Tables I and II). The penicillin G peaks most affected are those due to the aromatic and α-CH_2 groups, while the gem-dimethyl and C-3 proton peaks of ampicillin are also implicated.

The computer of average transients spectra indicate that the phospholipid peaks most affected are those due to the $(CH_2)_n$ and CH_3 groups. The line broadening observed is so marked in some cases (phosphatidylcholine–penicillin G and phosphatidylserine–penicillin G) that the signals are lost in the baseline.

Tables III and IV illustrate T_2 relaxation times for the antibiotic and phospholipid protons; they were determined by measuring the line width at half maximum peak height (31).

DISCUSSION

The following discussion has been limited to data relevant to penicillin–phospholipid interactions and does not attempt to discuss the previously documented details of the relaxation processes of proton lipid spectra previously referenced.

Previous work (9–26) showed that both the solid phospholipids and dispersions in water give NMR lines that are intermediate between solids and liquids at normal temperatures. A gradual narrowing of the lines is observed with rising temperature, and a "liquid" state can be observed in highly unsaturated phospholipids at temperatures normally found in biological systems. Sonication gives rise to a liquid signal from the hydrocarbon chain protons even when this is not present in the bulk material, indicating a substantial increase in chain mobility. The addition of substances like cholesterol and alamethicin removes the hydrocarbon chain signal, whereas other substances like procaine hydrochloride and epinephrine affect the hydrophilic parts of the phospholipids.

The antibiotics' spectra are relatively sharp, the chemical shifts being in accordance with published work (27, 30). The coupling constants (J) for the β-lactam doublets of penicillin G (Table I) are identical to those of Green *et al.* (30).

Comparison of the β-lactam signal for penicillin and ampicillin indicates that the two doublets in penicillin G collapse to give one singlet in ampicillin. The presence of an electron-attracting group such as an amino group may transfer its effect to the β-lactam ring to produce the observed differences. Pek *et al.* (32) found that substituents in the side chain affect the chemical shifts of the methyl protons remote from them. Recently, Kan *et al.* (29), using a 220-MHz. NMR spectrometer, reported that the phenyl signal and the —CH_2— signal of penicillin G are present as a multiplet and an *AB* quartet (J = 15 Hz.), respectively, whereas both appear as singlets at 60 and 100 MHz. Similarly, these workers reported two doublets for the β-lactam protons in dimethyl sulfoxide and that the —CH_A—CH_B—NH_X signals represent an *ABX* system with $J_{AX} = 0$, J_{AB} = 4 Hz., and J_{BX} = 9 Hz. The T_2 relaxation times for antibiotic protons have not been widely reported; however, the present findings (Table III) are of the same order as those of Fischer and Jardetzky (27).

[2] Koch-Light Laboratories Ltd.
[3] Merck.
[4] Fluorochem Ltd.
[5] Varian Associates HA-100.
[6] Varian Instruments Ltd.

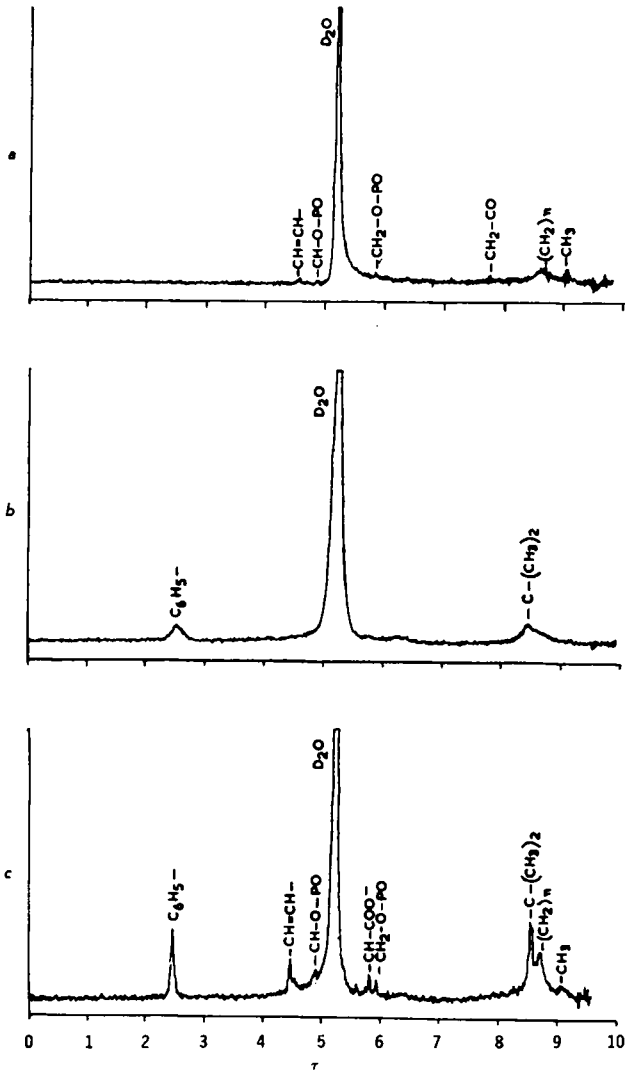


Figure 1—*The 100-MHz. NMR spectrum of 2% phosphatidylserine in deuterium oxide in the presence and absence of the antibiotics (2%). Key:* a, *phosphatidylserine;* b, *phosphatidylserine + penicillin G; and* c, *phosphatidylserine + ampicillin.*

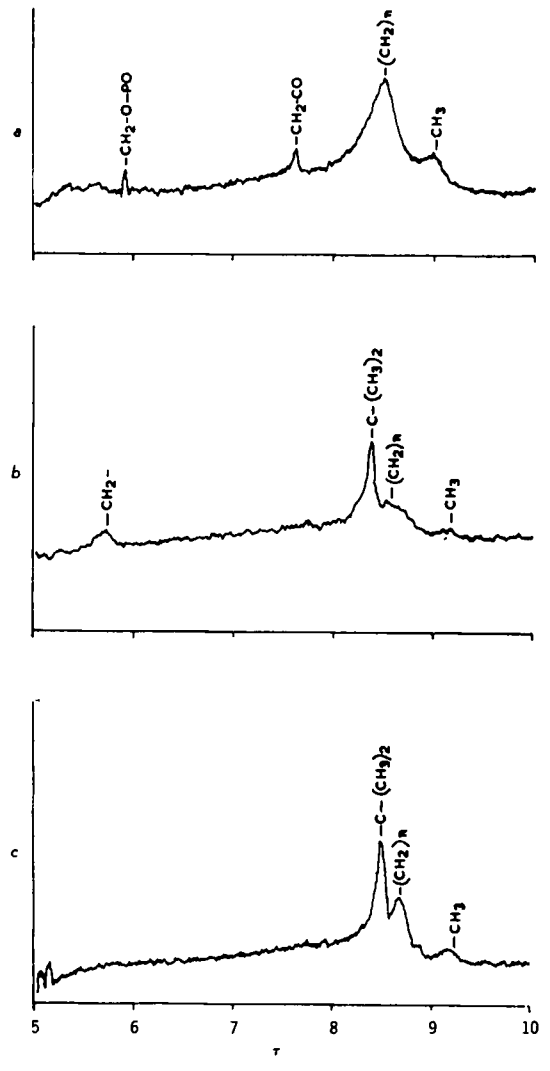


Figure 2—*The 100-MHz. NMR computer of average transients spectrum (15 scans) of 2% phosphatidylserine in deuterium oxide in the presence and absence of the antibiotics (2%). Key:* a, *phosphatidylserine;* b, *phosphatidylserine + penicillin G; and* c, *phosphatidylserine + ampicillin.*

The signals observed for the phospholipids (Table II) are in accordance with those of other workers. Sheard (18) indicated that only 88 ± 5% of the $^{+}N(CH_3)_3$ protons of III*a* contributed to the spectrum, behavior paralleled by the $(CH_2)_n$ protons. He also reported that this latter signal was more field dependent than the $^{+}N(CH_3)_3$ or CH_3 signals and proposed that each broad signal is a superimposition of many narrower signals.

The T_2 relaxation times for phospholipid protons have not been widely reported, but T_1 measurements (33) indicated a single relaxation time above and below the transition temperature, suggesting that a single relaxation mechanism exists for the bulk of the phosphatidylcholine molecule. The T_2 relaxation times found in this study are shown in Table IV.

Addition of the antibiotics to phospholipids indicates effects similar to those observed on addition of cholesterol (20, 24). Reduction in the motion of the fatty acid chain protons is so marked in some cases (penicillin G–phosphatidylcholine and penicillin G–phosphatidylserine) that the $(CH_2)_n$ and CH_3 signals are lost in the baseline (Table II).

In general, differential downfield line shifts are observed for the antibiotic protons, although those of penicillin G are more susceptible than those of ampicillin; the phospholipids, in contrast, experience an upfield shift. Downfield shifts indicate a deshielding effect, whereas shielding is implicated by upfield shifts. If the relative motion of the hydrocarbon chains is reduced by hydrophobic interaction with penicillin G, increased shielding of the protons is suggested and upfield shifts would be expected; examination of the spectra indicates this to be the case. It has been suggested (20) that the ends of the hydrocarbon chains are relatively more mobile than the rest of the chain, because the terminal CH_3 signal is broadened to a lesser extent than that representing the majority of the $(CH_2)_n$ protons.

More specifically, the phenyl and α-CH_2 protons of the antibiotics are shifted to a greater degree than other protons in the molecule, the effect being more pronounced for mixtures containing penicillin G. The phenyl and methylene protons become deshielded, possibly due to changes in the rotation of the side chain in the presence of phospholipids.

The greatest shifts of phospholipid protons observed are those present in the hydrocarbon chain, *i.e.*, $(CH_2)_n$ and CH_3. In mixtures of penicillin G–phosphatidylcholine and penicillin G–phosphatidylserine, these signals are lost in the baseline. However, due to the broad nature of these signals in the absence of the antibiotics, precise chemical shifts are not possible; a similar conclusion was reached by Hammes and Tallman (26).

The loss of the four peaks from the penicillin G spectra on addition of phosphatidylserine is interpreted as a case of extreme line broadening. In view of the observed changes in the phospholipid spectra, 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 antibiotic. Confirmation of this view was provided by substituting phosphatidylcholine for phosphatidyl-

Table II—Effect of Antibiotics on 100-MHz. NMR Signals of Phospholipid Protons in Deuterium Oxide (τ Values)

Phospholipid[a]–Antibiotic	CH_3	$(CH_2)_n$	CH_2—CO	$^+N(CH_3)_3$	CH_2—$^+N(CH_3)_3$	CH_2—OCO CH_2—OPO	CH—O—CO	CH=CH
PC	9.16	8.66	N/O	6.66	6.06	5.64	4.80	4.54
PC–penicillin G	N/O[b]	N/O	N/O	6.70	5.98	5.60	4.84	—[c]
PC–ampicillin	9.08	8.62	N/O	6.62	N/O	5.70	[d]	N/O
LPC	8.96	8.60	7.52	6.65	6.20	5.63	4.76	N/E
LPC–penicillin G	9.05	8.64	7.58	6.70	[e]	—[f]	[d]	N/E
LPC–ampicillin	9.04	8.62	7.54	6.79	6.20	5.57	N/O	N/E
PI	9.09	8.69	N/O	N/E	N/E	5.92	4.94	4.62
PI–penicillin G	9.08	8.71	N/O	N/E	N/E	5.85	4.98	4.70
PI–ampicillin	9.10	8.70	N/O	N/E	N/E	5.85	4.99	4.71
PS	9.03	8.64	7.75	N/E	N/E	5.84	4.88	4.57
PS–penicillin G[g]	N/O	8.70	N/O	N/E	N/E	5.68	N/O	N/O
PS–ampicillin	9.05	8.69	N/O	N/E	N/E	5.92	4.90	4.54

[a] Key: PC = phosphatidylcholine, LPC = lysophosphatidylcholine, PI = phosphatidylinositol, and PS = phosphatidylserine. [b] N/O = not observed, and N/E = not expected. [c] Under β-lactam signal. [d] Under spinning side band of deuterium oxide signal. [e] Merged with α-CH_2 signal. [f] Under C-3 signal. [g] Precipitation of this mixture.

Table III—Effect of Phospholipids on T_2 Relaxation Times of Antibiotic Protons in Deuterium Oxide (in Seconds)

Antibiotic–Phospholipid[a]	C_6H_5	α-CH—X[b]	C-6	C-5	C-3	C_2—$(CH_3)_2$	
Penicillin G	1.5	1.0	0.5	1.0	1.0	1.5	1.5
Penicillin G–PC	1.8	2.0	1.2	1.2	1.3	3.2	3.5
Penicillin G–LPC	1.0	1.3	1.0	1.0	1.0	1.2	1.2
Penicillin G–PI	1.8	1.3	1.1	1.0	1.0	1.3	1.3
Penicillin G–PS[c]	8.0		—	—	—		15.0
Ampicillin	2.0	0.75	1.0	1.0	0.5	1.2	1.2
Ampicillin–PC	1.5	1.5	1.0	1.0	0.8	1.3	1.3
Ampicillin–LPC	1.5	0.5	1.0	1.0	1.0	1.2	1.2
Ampicillin–PI	1.2	0.5	1.0	1.0	1.2	1.5	1.5
Ampicillin–PS	2.5	—[d]	1.5	1.5	1.5	4.0	4.0

[a] Key: PC = phosphatidylcholine, LPC = lysophosphatidylcholine, PI = phosphatidylinositol, and PS = phosphatidylserine. [b] X = H in penicillin G, and X = NH_2 in ampicillin. [c] Values given are peaks of very broad signals. No other signals. [d] Not observable under deuterium oxide peak.

serine when line shifts of 24 and 26 Hz. were recorded for the aromatic ring and α-CH_2 group, respectively (Table I). Ampicillin, being less hydrophobic than penicillin G (35), would be bound to a lesser extent; thus smaller changes were noted in the NMR spectra (Tables I and II). Rheological studies (3, 5) using hydrophobic bond breakers, such as urea and guanidine hydrochloride, indicated the hydrophobic nature of the antibiotic-phospholipid interaction.

Changes in T_2 relaxation times are more indicative of molecular changes than line shifts. Tables III and IV further implicate the parts of the antibiotic and phospholipid molecules already described. Some workers (26, 27) reported that a reduction in T_2 is indicative of restricted rotational freedom of certain groups stabilized by the interaction. However, where line broadening occurs, one may observe an increased value of T_2, suggesting greater freedom; this occurs because the line becomes so broad that the area under the peak is the same as or greater than the peak area of the signal before interaction.

Complex formation can often be detected by observing the changes in chemical shift or relaxation time of a signal; the former is due to changes in charge density at a given proton resulting from interaction. However, line broadening may result even though no change in charge density is observed.

Line broadening may be due to magnetic inhomogeneity, viscosity changes, self-association, or intermolecular complex formation. Viscosity can be disregarded because a 2% solution of phosphatidylcholine has a low viscosity (1.0260×10^{-2} poise at 30°). Sharp resonance lines, although broadened, were observed for the antibiotic protons, thus ruling out magnetic inhomogeneity. Self-association of penicillin G has been reported (34); but since line broadening of some phospholipid signals is also observed, self-association

Table IV—Effect of Antibiotics on T_2 Relaxation Times of Phospholipid Protons in Deuterium Oxide (in Seconds)

Phospholipid[a]–Antibiotic	CH_3	$(CH_2)_n$	CH_2—CO	$^+N(CH_3)_3$	CH_2—$^+N(CH_3)_3$	CH_2—O—CO CH_2—O—PO	CH—O—CO	CH—CH
PC	22.0	30.0	N/O	28.0	10.0	5.5	4.0	4.0
PC–penicillin G	N/O[b]	N/O	N/O	17.0	2.5	2.4	2.6	—[c]
PC–ampicillin	17.0	20.5	N/O	6.0	N/O	1.6	—[d]	N/O
LPC	9.0	8.0	9.0	3.2	10.5	1.5	1.0	N/E
LPC–penicillin G	10.0	11.0	12.0	4.3	—[e]	—[f]	[d]	N/E
LPC–ampicillin	9.0	12.0	16.0	4.8	11.5	2.0	N/O	N/E
PI	15.5	22.0	N/O	N/E	N/E	3.5	2.0	2.0
PI–penicillin G	13.0	19.0	N/O	N/E	N/E	2.0	3.0	2.2
PI–ampicillin	13.0	16.0	N/O	N/E	N/E	4.5	2.0	2.0
PS	15.0	26.5	3.5	N/E	N/E	2.5	2.5	4.0
PS–penicillin G	N/O	43.0	N/O	N/E	N/E	12.0	N/O	N/O
PS–ampicillin	15.0	22.0	N/O	N/E	N/E	4.5	4.5	—[c]

[a] Key: PC = phosphatidylcholine, LPC = lysophosphatidylcholine, PI = phosphatidylinositol, and PS = phosphatidylserine. [b] N/O = not observed, and N/E = not expected. [c] Under β-lactam signal. [d] Under spinning side band of deuterium oxide signal. [e] Merged with α-CH_2 signal. [f] Under C-3 signal.

of the antibiotic is unlikely to be the only mechanism, if it occurs at all (5).

The experimental evidence indicates rapid exchange of penicillin G between the free and unbound forms; if the exchange was slow, sharp lines due to the free species would be superimposed on the broad lines.

Thus, a complex appears to be formed between the antibiotics and phospholipids. This may involve the side chain of the antibiotics and fatty acid chains of the phospholipids. The change in peak size of the $(CH_2)_n$ and CH_3 protons indicates changes in the relative mobility of the hydrocarbon chains. In all phospholipid dispersions examined, penicillin G was observed to interact to a greater degree than ampicillin, while the phospholipids interacted in the order phosphatidylserine > phosphatidylcholine > phosphatidylinositol > lysophosphatidylcholine. The significance of the results in relation to the differences in activity of these antibiotics was discussed elsewhere (1, 5).

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